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PESTICIDE INDUCED DELAYED NEUROTOXICITY
Proceedings of a Conference

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16. ABSTRACT

A delayed neurotoxicity syndrome, shown to be induced by certain organophosphorus esters, was first reported almost 50 years ago. The 1975 Guidelines for Registering Pesticides in the United States reflect a continuing concern for this problem in the recommendations made for evaluating the delayed neurotoxicity hazard associated with organophosphate pesticide use.

This conference was designed to bring together those individuals interested in the organophosphorus-ester-induced neurotoxicity syndrome to stimulate discussions and to evaluate the latest scientific results. With the aid of formal and informal presentations, new pathways for investigation are becoming apparent which may lead to the understanding and elimination of this problem.

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Proceedings of a Conference
February 19-20, 1976
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Sponsored by:

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U. S. ENVIRONMENTAL PROTECTION AGENCY
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ACKNOWLEDGEMENTS

The objective of the Symposium was to bring to a specific open forum a problem that has been recognized and investigated intensively for almost half a century. It was hoped that the presentations and discussions would stimulate new research to fully understand and control the problem. This document is a compilation of the papers presented and the discussion of the two day meeting.

There are many people to whom the EPA and NIEHS are indebted for their work in putting this meeting together. Thanks are due to the participants for attending and special acknowledgements are due Dr. William Upholt and Dr. John Casida for taking the time to serve as chairmen. In preparation for the meeting, the following were instrumental in planning the agenda: Dr. William Durham, Dr. Orville Paynter, Mr. August Curley, Dr. John Casida, and Dr. Florence Kinoshita. Additional thanks are due to Mrs. Sandra Townsend of Kappa Systems, Inc. and Mr. Frank Ayers of the Research Triangle Institute for their excellent management of the planning sessions and the execution of the program.

I greatly acknowledge the help of all those responsible for reading and putting together the final document. Special thanks are due to Mrs. Donna Wicker for her assistance with the revisions and corrections that were necessary. Without all of the above people, this meeting could not have taken place.

Ronald L. Baron

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INTRODUCTION

DR. TERRI DAMSTRA

Ladies and Gentlemen, welcome to the conference, Pesticide-Induced Delayed Neurotoxicity, jointly sponsored by the U. S. Environmental Protection Agency and the National Institute of Environmental Health Sciences.

Recent investigations, have provided some exciting insights into the possible mechanisms of action of organophosphorous esters induced delayed neurotoxicity. We will hear about some of the primary biochemical events, pathology, structure activity relationships, effects on the EEG, and medical treatment. Hopefully, some of these beginning insights into the mechanisms of action will also provide some very practical benefits, such as the development of better test systems for the accurate prediction of the potential of delayed neurotoxicity of pesticides in both agricultural and industrial work places.

I'm sure that the presentations of the papers will stimulate a great deal of discussion. We hope that the conference will at least generate some new ideas and approaches that are necessary to continue the advancement of both the understanding and elimination of the problem of delayed neurotoxicity.

Once again, welcome, on behalf of the Environmental Protection Agency, and the National Institute of Environmental Health Sciences.

DEDICATION

The EPA consultant group that met in the summer of 1975 with the responsibility of planning this Symposium was unanimous in proposing that Dr. John M. Barnes of the Medical Research Council Laboratories, Carshalton, England was the most appropriate scientist to present the introduction on Recognition and Overview of the Organophosphorus Delayed Neurotoxicity Problem. This was a very easy and logical choice on considering the major contributions Dr. Barnes made to this and many other areas of toxicology. With the death of Dr. Barnes on September 24, 1975, this Symposium and the scientific community as a whole suffered a severe loss. Dr. Barnes made a tremendous number of significant contributions to science and was a renowned leader in the field of toxicology. The neurotoxic syndrome which is the subject of this Symposium was researched intensively by Dr. Barnes. His contributions stimulated much of the work reported to date. I suggest that this Symposium be dedicated to the memory of Dr. John M. Barnes with the hope that the work he so ably began in many areas will continue for the benefit of the peoples of all nations.

Dr. R. L. Baron

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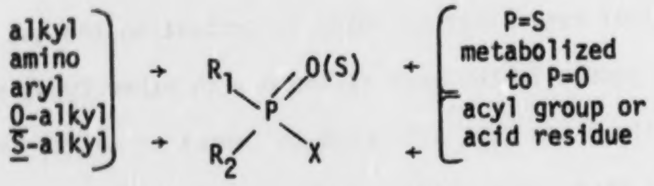
RECOGNITION AND OVERVIEW OF THE ORGANOPHOSPHORUS INDUCED DELAYED NEUROTOXICITY PROBLEM

John E. Casida, Ph.D. and Ronald L. Baron, Ph.D.

Organophosphorus Biocides

Organophosphorus (OP) esters are now the major chemical weapons used to combat pest insects and acarines of agricultural, veterinary and medical importance. More than 100 OP biocides are currently in use of which one or two dozen are major compounds. They collectively account for an annual worldwide production exceeding 200 million (2×10^8) pounds.

The insecticidal activity of OP compounds was discovered in 1937 by Gerhard Schrader who was instrumental in deriving a type formula for the biocidal structures. They are acid anhydrides of the following type:



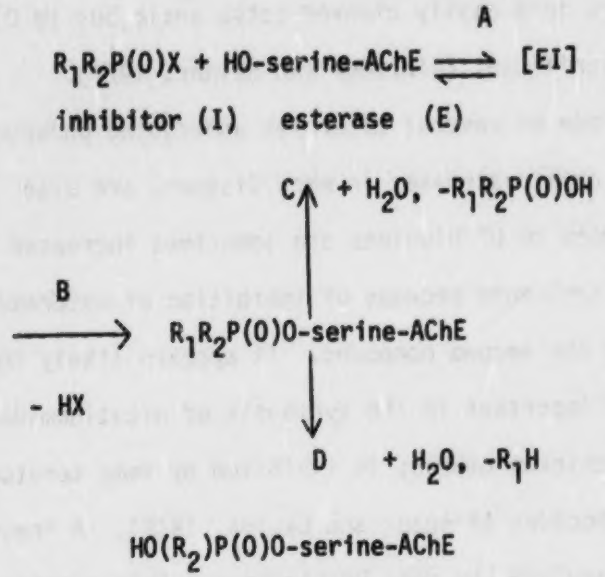
The P=S compounds are metabolically oxidized to the P=O derivatives before they are toxic. The first important OP insecticides and acaricides were O,O-dimethyl or O,O-diethyl phosphates (P=O) or phosphorothionates (P=S) optimized for high contact potency by varying the acyl group. Subsequent developments led to the introduction of plant and animal systemics, i.e. compounds absorbed and translocated in insecticidal concentrations by crops and livestock.

In the course of testing many tens of thousands of OP compounds to optimize the insecticidal activity and reduce the hazard for mammals (i.e. selective toxicity), other types of useful biocidal activity were discovered, extensively studied, and often reduced to practice. Thus, the OP compounds include interesting and/or important nematocides, rodenticides, avicides, insect chemosterilants and insecticide synergists, fungicides, herbicides and plant growth regulators. These new compounds of widely divergent structures introduced new problems. With the reduced mammalian toxicity, there was a greater chance of demonstrable secondary effects at nonlethal dosages. With compounds acting on plants and microorganisms, emphasis was placed on the induction of biochemical lesions of different types than that associated with the insecticidal and acaricidal activity. Thus, man and his environment are now exposed to OP compounds that have the potential for inducing a variety of biochemical lesions (Eto, 1974).

The toxicological tests that are made in evaluating the use safety of OP biocides are generally the same as those with other types of toxicants, especially those that are found to impact on man as inadvertant food additives. In part, they include acute toxicity studies, life-time feeding studies in at least 2 mammalian species, metabolic and biochemical studies, and an evaluation of carcinogenic, mutagenic and teratogenic potential.

Toxicology of Organophosphorus Compounds

The primary parasympathomimetic effects of the OP insecticides and acaricides, in both the pest organisms and in mammals, are attributable in part or entirely to phosphorylation of a serine residue within the active site of acetylcholinesterase (AChE), a critical enzyme in the nervous system. The simplified reaction sequence is as follows:



$$\begin{tikzcd}
R_1R_2P(O)X + \text{HO-serine-AChE} \arrow[r, shift left, "A"] & \text{[EI]} \arrow[l, shift left] \\
\text{inhibitor (I)} \quad \text{esterase (E)} & \\
 & \text{C} + H_2O,\ -R_1R_2P(O)OH \\
{} \arrow[r, "B", "-\ HX"'] & R_1R_2P(O)O\text{-serine-AChE} \arrow[u] \arrow[d] \\
 & \text{D} + H_2O,\ -R_1H \\
 & HO(R_2)P(O)O\text{-serine-AChE}
\end{tikzcd}$$

In the first step (A), the OP inhibitor (I) combines with the esterase (E) to form an esterase-inhibitor complex (EI). With a suitable fit at the active site of AChE and sufficient reactivity of the OP compound, a serine residue critical for the esteratic activity undergoes phosphorylation (B). The inactive phosphorylated enzyme which is formed normally cleaves by dephosphorylation (C) to regenerate the active esterase. However, a portion of the inhibited esterase may undergo cleavage of the R_1-P bond (D) in a process referred to as "ageing", yielding an inactive enzyme with a very stable ionized acidic phosphorus group on the esteratic site. The rate of each reaction is strongly dependent on the configuration and reactivity of R_1,R_2 and X on the nature and conformation of the active site of the esterase. Other AChE inhibitors such as carbamates [$R_1R_2NC(O)X$] and sulfonates (RSO_2X) react in steps A, B and C in an analogous manner. However, the inhibited derivatives are more easily cleaved (step analogous to C) and reaction D is not significant (Aldridge and Reiner, 1972).

Nerve AChE is only one of several esterases undergoing phosphorylation. Other forms of cholinesterase, in many tissues, are also inhibited. Animals exposed to OP biocides are sometimes increased in susceptibility to other toxicants because of inhibition of esterases important in detoxifying the second compound. It appears likely that a "teratogenic esterase" important in the synthesis of nicotinamide-adenine dinucleotide in chicken embryos is inhibited by some teratogenic OP and methylcarbamate biocides (Proctor and Casida, 1975). A "neurotoxic esterase" is also phosphorylated by some OP biocides. This reaction is central to the subject of delayed neurotoxicity and is considered in detail in later contributions to this Symposium.

Most but not all OP biocides act as phosphorylating agents. Others are mild to potent alkylating or acylating agents. The fungicidal activity of some OP compounds appears to result from alkylation or acylation. Many OP biocides react readily in mammals with glutathione, catalyzed by glutathione S-transferase; the alkylation or arylation of glutathione probably serves as a protective mechanism against more deleterious lesions. The alkylating activity is also of interest in relation to possible mutagenic and carcinogenic effects of OP compounds. The OP insect chemosterilants, such as aziridinyl phosphorus compounds, alter nucleic acid metabolism, but these chemosterilants are not used because of the hazards associated with such action. The alkylating activity of OP insecticides is an area of definite interest and concern, and one of a considerable volume of research literature (Eto, 1974).

There are also other actions of OP biocides, two of which warrant mention. Many phosphorothionates and O-(2-propynyl) phenylphosphonates are potent but transient in vivo inhibitors of hepatic microsomal mixed-function oxidases important in zenobiotic metabolism (Casida, 1970; De Matteis, 1974; Norman et al., 1974). Some extremely toxic 4-alkyl-1-phospha-2,6,7-trioxabicyclo[2.2.2]octane derivatives act as central nervous system stimulants, possibly by blocking inhibitory neuronal mechanisms (Casida, et al., 1976). These actions are not dependent on the phosphorus substituent since non-phosphorus analogs are also active.

This background on the deversity of actions of OP biocides serves to point out the complexity of problems in evaluating their toxicology. It also serves to focus on the subject of greatest immediate interest and concern, that of delayed neurotoxicity.

Delayed Neurotoxicity Induced by Chemical and Microbial Agents

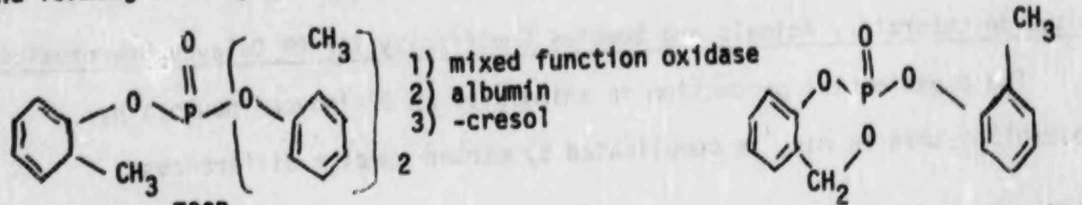
Exposure to a wide variety of chemical and biological agents leads to injury of the human nervous system involving axonal and/or myelin disruption. Toxic neuropathies result from, among other causes, exposures to the following agents: inorganic chemicals (especially salts of heavy metals such as arsenic, thallium, mercury or lead); drugs [isoniazid, p-bromophenylacetylurea, hydralazine, L-penicillamine, tetraethylthiuramdisulfide (antabuse), the nitrofuran group (nitrofurazone, nitrofurantoin, furaltadone), clioquinol, plasmocid, distavol, thalidomide and several vinca alkaloids]; fungicides (diethyldithiocarbamates); solvents (carbon disulfide, methylbutylketone, trichloroethylene, hexane); polymer or plasticizer precursors (acrylamide, OP compounds); many biological agents including viruses, toxins, etc. (Cavanagh, 1964, 1973). Other neuropathological conditions resulting in human central or peripheral nervous system disruption include such disorders as: amyotrophic lateral sclerosis; Friedreichs ataxia; Werdnig-Hoffmann's disease; certain vitamin deficiencies; and acute porphyria neuropathy.

Some OP biocides also have the potential to produce delayed neurotoxicity. This subject will be reviewed briefly here and in detail in later Symposium papers.

Tri-o-Cresyl Phosphate (TOCP) and OP Delayed Neurotoxicity

A most unfortunate incident involving more than 20,000 human cases of peripheral neuropathy occurred in the United States around 1930. Researchers in the U. S. Public Health Service identified TOCP as the etiological agent (Smith et al., 1930), for the first time focusing attention on OP delayed neurotoxicity. In this instance, the syndrome was referred to initially as "ginger paralysis" or "ginger jake" denoting the source of beverage adulteration ("Jamaica Ginger") during prohibition that led to the ataxia. The clinical signs were characterized as bilateral and symmetrical flaccid paralysis of the distal muscles predominantly of the lower extremities. Histological examination of central and peripheral nerve tissue preparations from early animal studies revealed extensive disruption of the myelin sheath, suggesting a process of demyelination as the principal mechanism leading to ataxia. The term "demyelination" denotes the end result of a step-wise process involving sequential block of an undefined biochemical reaction, axonal disruption presumably starting at the distal portion, a dying-back process, and finally myelin disruption. Clinical signs of this neurotoxic syndrome are produced in man by a dose within the range of 2 to 40 mg TOCP/kg. The second catastrophic incident of TOCP neurotoxicity occurred in Morocco in 1959 as the result of tricresyl phosphate from a lubricating oil normally used in jet engines containing known amounts of TOCP which had been mixed with olive oil for human consumption (Smith and Spalding, 1959). The "ginger jake" and Morocco fiascoes account for most of the more than 40,000 known cases of OP delayed neurotoxicity in man. Incidents of this type with TOCP, fortunately on a small scale, continue to appear periodically.

TOCP requires bioactivation prior to exerting its neurotoxic effect. The conversion of TOCP to a neurotoxic agent is due to a two-step reaction, the first step involving mixed-function oxidase hydroxylation of a methyl group to form a hydroxy intermediate and the second a cyclization, catalyzed by albumin, liberating a cresyl group and forming a saligenin cyclic phosphate.

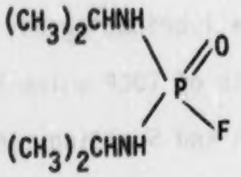


TOCP

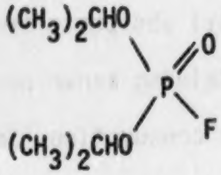
saligenin cyclic phosphate
(the neurotoxic metabolite)

TOCP continues to be used in laboratory experiments as the model compound against which the effects of other materials are measured. The saligenin cyclic phosphate metabolite and related compounds are used in studies of the biochemical lesion.

Accidental poisoning with one candidate pesticide chemical (the systemic insecticide Mipafox) resulted in three cases of human delayed neurotoxicity (Bidstrup et al., 1953). This compound is closely related to DFP, another chemical normally used in laboratory neurotoxicity studies.



Mipafox



DFP

To date, no case of human neurotoxic involvement has been reported as the result of recognized and authorized industrial and agricultural use of OP compounds. However, accidental poisoning and gross misuse may lead to severe consequences. It is clear that new OP compounds must be evaluated for potency as delayed neurotoxins prior to their commercial use as industrial or agricultural chemicals (Davies, 1963; Johnson, 1975a,b).

Tests on Laboratory Animals and Species Specificity for OP Delayed Neurotoxicity

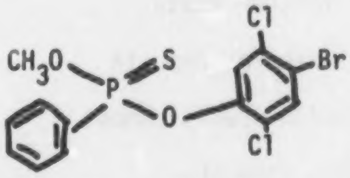
The experimental production in animals of an OP-induced neuropathy, resembling that in man, is complicated by marked species differences in susceptibility and clinical signs. The chicken appears to respond most consistently in mimicing the dose- and time-dependent clinical signs of human poisoning. Because of their availability, and ease of adaptation to laboratory conditions, hens are routinely used as the test animals with TOCP as the standard reference compound. The clinical signs after acute TOCP administration to hens are such that the birds appear normal for a latent period of 8 to 14 days; they then spend an increasing proportion of time sitting in a characteristic position and, when exercising, they display a clumsiness of gait accompanied by overt weakness; by 15 to 20 days after treatment the birds are severely paralyzed in the legs and lower extremities and the wings are detectably weakened. At low neurotoxic doses, a weight loss observed over the 3-week period will be reversed and the animal will maintain general good health although not fully regain the use of its affected appendages. At doses producing a severe clinical neuropathy, the animal loses its ability to eat and subsequently dies following a severe weight loss.

There are fascinating and disturbing species differences in the OP delayed neurotoxicity. In man and mature hens a single acute oral administration is sufficient to initiate the irreversible clinical response and these species are also susceptible to subacute poisoning. There is no distinct response in young hens and variable responses in non-human primates (squirrel and Rhesus monkey and baboon). In rats, rabbits and other rodent species, the delayed signs of poisoning are not readily induced. In this case TOCP administration yields cholinergic signs of poisoning which are subsequently replaced within variable time intervals by flaccid paralysis of musculature followed by respiratory paralysis and death. The guinea pig develops a delayed ataxic response on extended subacute administration of DFP. In dogs, which are a sensitive species, TOCP and DFP induce flaccid paralysis of the hind quarters but only after an unusually extended delay period. Other susceptible species include the cat, calf and cow, sheep and horse. It is unfortunate that atypical responses are obtained with rats and dogs, species used routinely in life-time feeding studies. In considering the results of tests with laboratory animals it must be borne in mind that man may be more sensitive than any species thus far examined, at least to TOCP-induced delayed neurotoxicity (Davies, 1963; Johnson, 1975 a,b).

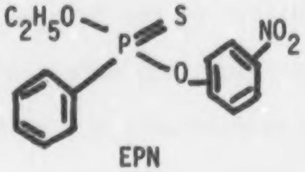
Structure-Activity in Relation to the Biochemical Lesion

It is extremely important to understand the relationship of chemical structure and neurotoxicity, at both the organismal and biochemical levels. It is clear that many of the delayed neurotoxins require bioactivation (see above for TOCP) and that phosphorylation of a critical nerve esterase is involved. The most extensive and successful investigations on the biochemical lesion have been carried out by Johnson, Aldridge and Barnes of the Medical Research Council Laboratories, Carshalton, England (Johnson, 1975 a,b). Johnson has shown by an elegant series of *in vivo* and *in vitro* experiments with hens that inhibition of a specific nerve esterase, the "neurotoxic esterase", is associated with the delayed neurotoxicity. Further, he has proposed a simple *in vitro* enzyme assay to determine whether or not a new OP compound is likely to induce delayed neurotoxicity. These findings are beginning to clarify the seemingly confusing relationship of chemical structure and delayed neurotoxicity. They may ultimately provide a basis for understanding the species variations in response, which might result from different sensitivities of the relevant enzymes or varying significance of the inhibited pathways in maintaining normal nerve function. Other workers have examined, with little or no success, the possible involvement in the delayed neurotoxicity of various vitamins (nicotinic acid, thiamine and tocopherol), steroids, cortisone, lipids, metals (copper) and ceruloplasmin (Davies, 1963; Johnson, 1975a,b; Kimmerle and Loser, 1974).

The following pesticides (plus carbophenothion and trichlorfon) that are currently in use or that have been subjected to widespread trials are reported to have delayed neurotoxic properties on single or multiple dose administration to hens:



leptophos (Phosvel)



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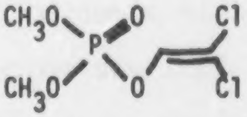
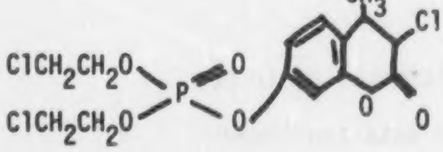
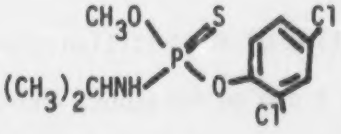


dichlorvos



Haloxon



DMPA (Zytron)

$[CH_3(CH_2)_3S]_3P(O)$

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$[CH_3(CH_2)_3S]_3P$

Merphos

Many important OP biocides without delayed neurotoxic properties are closely related to neurotoxic compounds (Eto, 1974; Johnson, 1975a,b). A large number of very effective candidate pesticides have been dropped by industry when their neurotoxic properties were discovered. Thus, regulatory aspects are an important part of the consideration of OP delayed neurotoxicity.

Regulatory Aspects of OP Biocides with Delayed Neurotoxic Activity

The year 1975 and the OP insecticide leptophos, which is registered in several countries for use on cotton and food crops, have focused attention on the regulatory aspects of OP delayed neurotoxicity. Two actions must be considered, one by the United States EPA and the other by a World Health Organization/Food and Agriculture Organization (WHO/FAO) Committee.

In mid-1975 the EPA issued a notice of proposed revocation of tolerances previously established for leptophos (Ritch, 1975). Initial neurotoxicity studies with leptophos were negative but further tests showed the propensity for initiating the delayed neurotoxic response in hens. In a move designed to insure the safety of man and his environment and to assure that residues in food will not be hazardous, the EPA proposed the revocation of tolerances for leptophos. The notice of revocation stated:

> "...a reevaluation of the petition (for tolerances of 10 ppm on lettuce and 2 ppm on tomatoes) and other data confirmed that leptophos is an agent which produces delayed neurotoxicity in hens. Additional information on leptophos is

> necessary to evaluate the possible hazard to man and other nontarget species from the potential effects of its use. Based on the above information, there is a reasonable basis to propose revocation of the established tolerances for residues until adequate data are provided by the petitioner to show that the use of leptophos on lettuce and tomatoes will not be detrimental to the public health."

This appears to be the first time a pesticide tolerance established by a U. S. regulatory authority has been recommended for revocation.

Also in 1975, the WHO Expert Committee on Pesticide Residues in a Joint Meeting with the FAO Working Party of Experts on Pesticide Residues considered the problem of delayed neurotoxicity, in conjunction with an examination of the worldwide use of leptophos in agriculture and with respect to its safety as a residue in food entering international commerce. This consideration was in conjunction with the role of the Joint Meeting in estimating an Acceptable Daily Intake (ADI) and recommending maximum residue limits (tolerances) in food to the Codex Alimentarious Commission. The Joint Meeting considered that:

> "the potential hazards associated with delayed neurotoxicity are two-fold: (1) exposure of occupationally or accidentally exposed individuals who would be affected by high doses for short periods; and (2) long term low level exposure and possible build-up of the toxicant to threshold levels leading to ataxia. Although the first principle does not fall into the direct terms of reference of the Meeting, the toxicological

hazard associated with such exposure must be considered. The Meeting considered that delayed neurotoxicity is a parameter of toxicology that appears to follow a dose-response relationship and as such a no-effect level could be estimated following acute or chronic exposure in a susceptible species. With an adequate margin of safety, an ADI for man can be allocated as far as pesticide residues in food are concerned, with a sufficient degree of assurance."

The WHO/FAO Committee took a very significant stand on the problem of delayed neurotoxicity. They concluded that OP-induced delayed neurotoxicity is a toxicological syndrome similar to other toxicological parameters and subject to the same quantitative considerations given other types of toxicants. The dosage-response relationship was considered to be applicable to delayed neurotoxicity as it is to other modalities of injury. The WHO/FAO Joint Meeting reported that sufficient toxicological data had been presented for leptophos, including short and long-term studies with rats and dogs, reproduction investigations, and carcinogenesis studies. The only significant adverse toxicological parameters reported were pup mortality at high levels in the reproduction study and delayed neurotoxicity in hens. Based on an observed no-effect level in rats and dogs and the no-effect level for delayed neurotoxicity in acute studies with hens, a temporary ADI of 0 - 0.001 mg/kg body weight was established. This group reflected its concern over the delayed neurotoxicity finding by applying a high safety factor to the experimental no-effect levels in animals in arriving at the ADI for man. In conjunction with the establishment of an ADI, the WHO/FAO Joint Meeting recommended the establishment of tolerances on 20 crops with values ranging from 0.05 to 2 mg/kg (ppm). These recommendations will now be considered by the Codex Alimentarius Commission with respect to establishing international tolerances for world trade.

While the two actions taken in 1975 (by the EPA and the WHO/FAO Joint Meeting) may appear to be in conflict, they reflect the continuing difficulties in evaluating the safety of toxicants and their direct and indirect effect on man and his environment. The validity and implications of these actions with respect to world trade will be discussed for many years in select circles. This Symposium deals with both the basic science and regulatory aspects of OP-induced delayed neurotoxicity. They are critical and timely issues for the future of OP biocides and the establishment of adequate safety standards.

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REACTION OF SERINE ESTERASES RELEVANT TO THE DELAYED NEUROTOXICITY PROBLEMS

W. N. Aldridge, Ph.D.

This talk must be regarded as an introduction to Dr. Johnson's lecture on the mechanism of delayed neurotoxicity. I shall discuss very briefly those properties of the reaction of organophosphorus compounds with esterases which have been used in the development of his hypothesis. Some of this no doubt will be to many of you old information but the emphasis will be directed towards the above purpose. The first part concerns the reaction which seems to be an exact analogy of the substrate - enzyme system. The aging reaction which has no such analogy will be dealt with in more detail. (See Figure 1)

Evidence has been obtained for all the steps shown in the figure. For most examples the rate of reaction of organophosphorus compounds with esterase follows 1st order kinetics and the rate of the reaction is directly proportional to the concentration of inhibitor. (See Figures 2 and 3)

In some instances there is indirect evidence for saturation kinetics. Thus, the evidence for substrates of the existence of a Michaelis type complex is that a maximum rate of product formation is reached - i.e. when the enzyme is all present as the reversible complex with the substrate. In a similar way for some organophosphorus compounds the first order constant for inhibition is not linearly related to the concentration of inhibitor and the rate tends towards a maximum. This

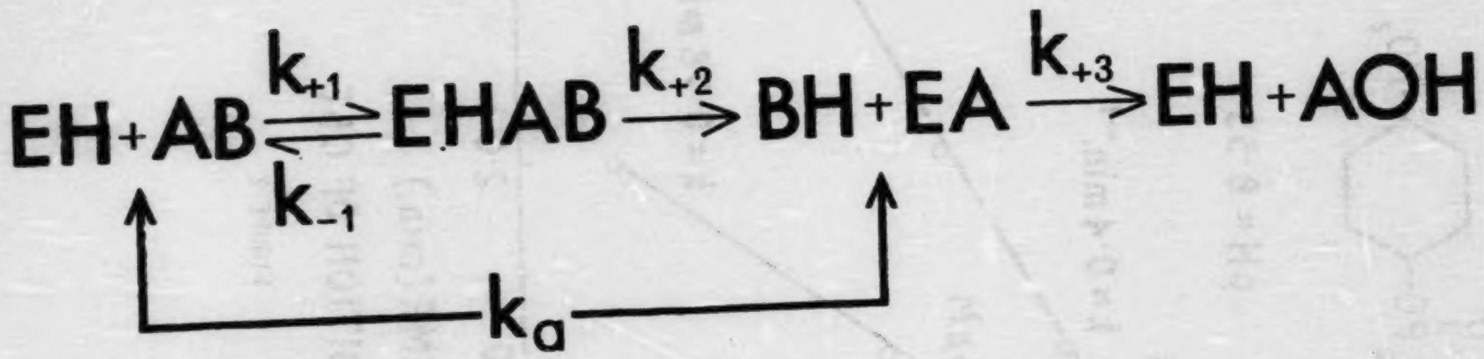
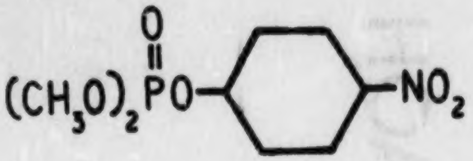
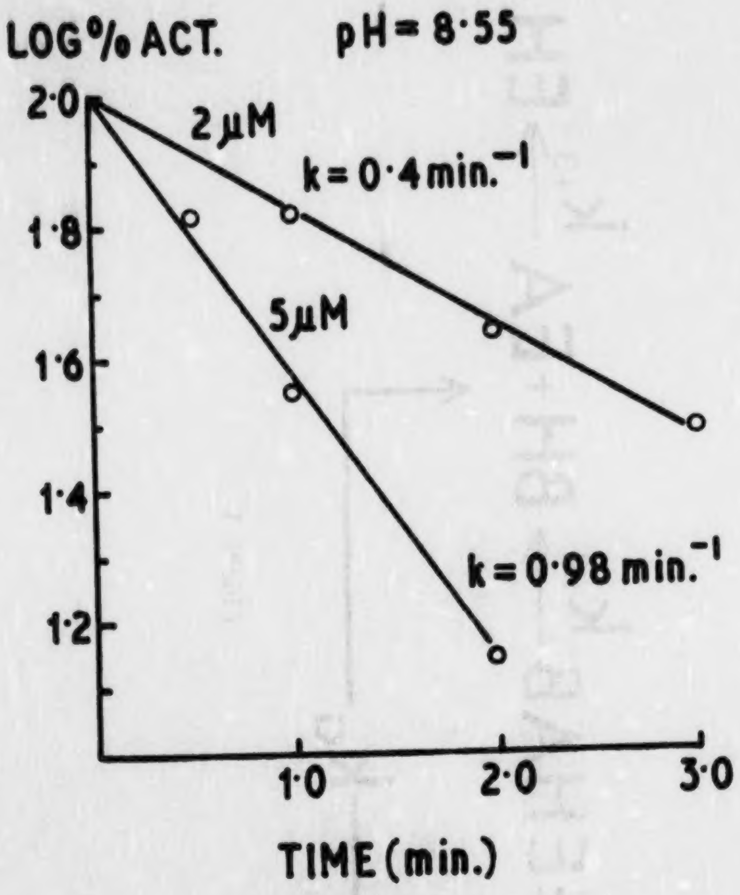

EH + AB
k_{+1}
k_{-1}
EHAB
k_{+2}
BH + EA
k_{+3}
EH + AOH
k_a

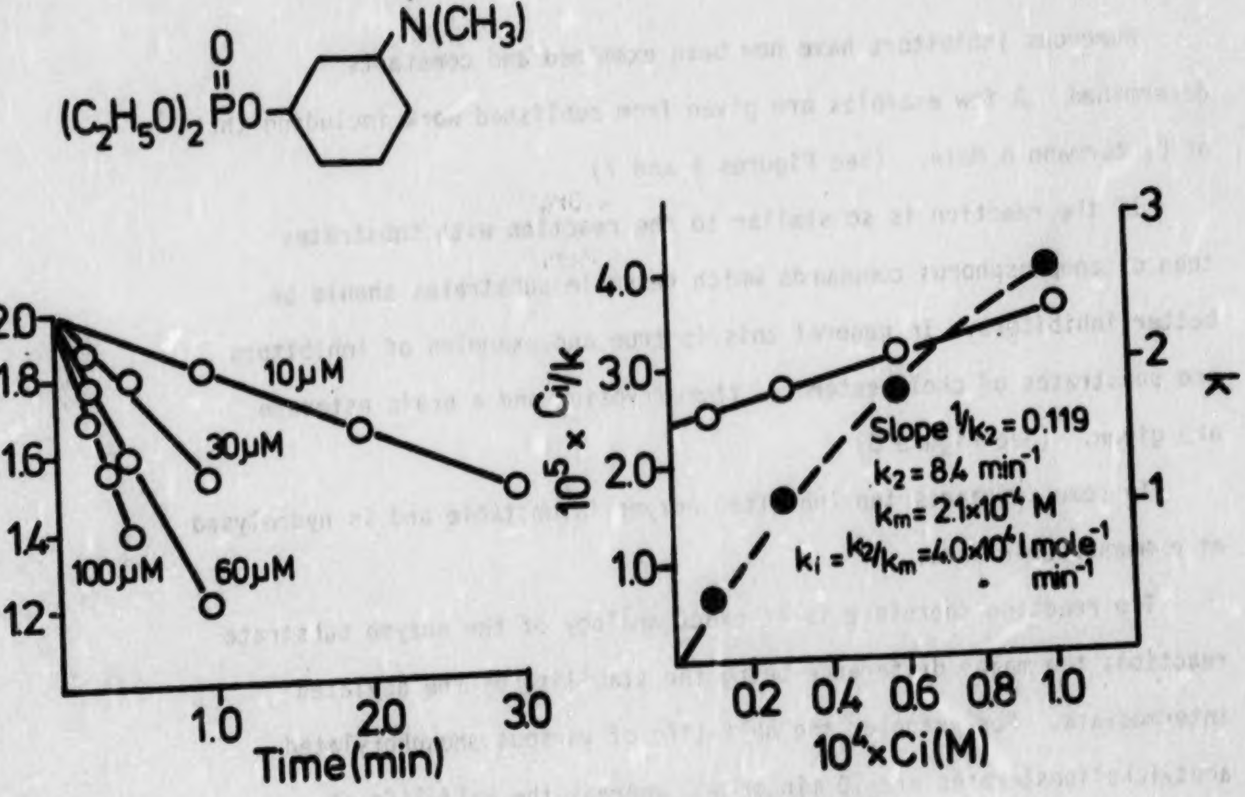
FIGURE 1





INHIBITION OF ChE

FIGURE 2



Inhibition of ChE at pH 8.9

FIGURE 3

is interpreted as evidence for the existence of a reversible Michaelis-type complex between inhibitor and enzyme. (See Figure 4)

When this condition is found the bimolecular rate k_a constant is not determined but K_a and k_{+2} may be separately measured. (See Figure 5)

Numerous inhibitors have now been examined and constants determined. A few examples are given from published work including that of Dautermann & Main. (See Figures 6 and 7)

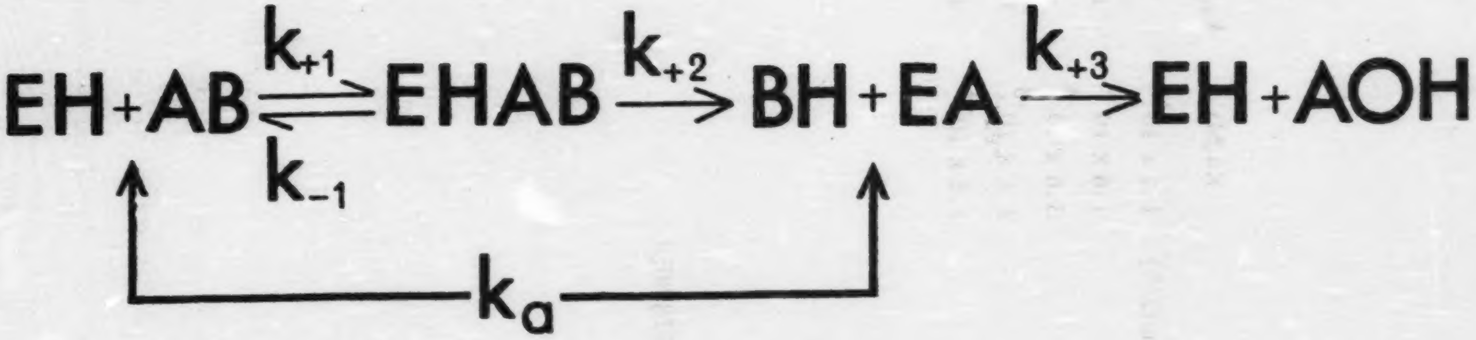
If the reaction is so similar to the reaction with substrates then organophosphorus compounds which resemble substrates should be better inhibitors. In general this is true and examples of inhibitors and substrates of cholinesterase, chymotrypsin, and a brain esterase are given. (See Figure 8)

In some instances the inhibited enzyme is unstable and is hydrolysed at a measureable rate.

The reaction therefore is an exact analogy of the enzyme substrate reaction, the major difference being the stability of the acylated intermediate. For example, the half-life of various phosphorylated acetylcholinesterases are 10 min or so, whereas the half-life of acetylated cholinesterase, the intermediate in the hydrolysis of acetylcholine, is approximately 100 μ sec. (See Figure 9)

The rate of reaction of organophosphorus compounds is influenced by many factors, and these are outlined in the figure. (See Figure 10)

The fact, that the inhibited enzyme is normally stable and the inhibition follows defined kinetics, has allowed different esterases

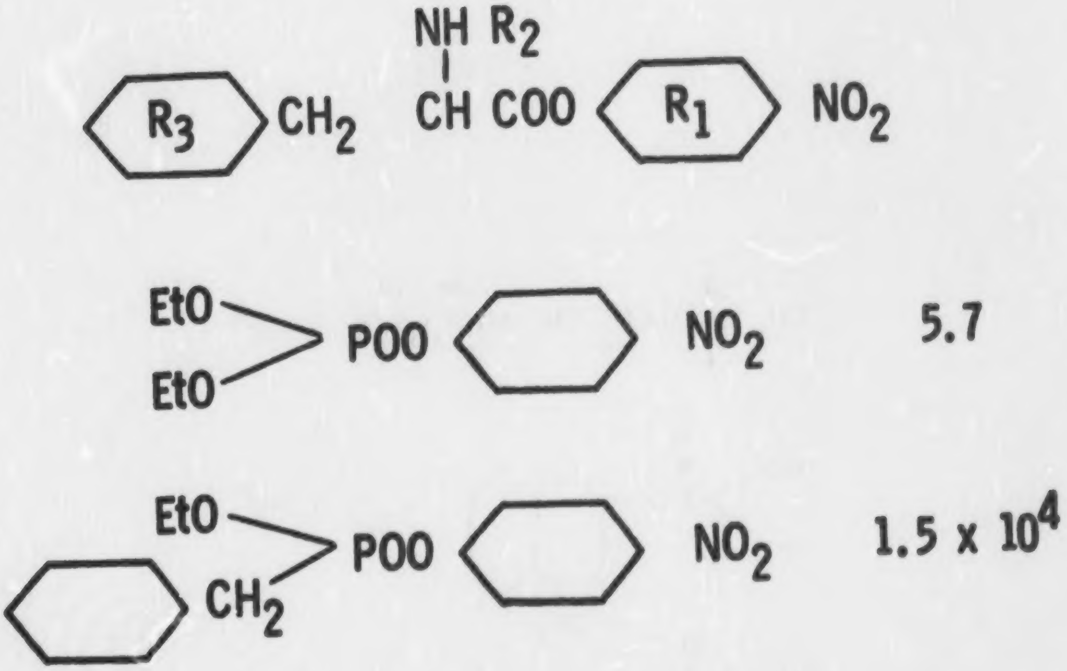


$$\mathrm{EH} + \mathrm{AB} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{EHAB} \xrightarrow{k_{+2}} \mathrm{BH} + \mathrm{EA} \xrightarrow{k_{+3}} \mathrm{EH} + \mathrm{AOH}$$

FIGURE 4

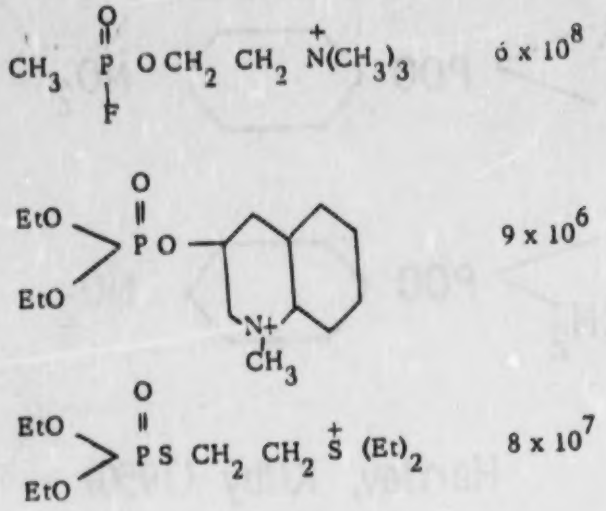
	Ka(M)	$k_{+2}(\text{min}^{-1})$
Phosphostigmine(25°)	1.2×10^{-4}	5.2
DFP (5°)	1.6×10^{-3}	11.9
Paraoxon (5°)	3.6×10^{-4}	42.7
Malaoxon(5°)	2.4×10^{-3}	67.0
Tetram(5°)	1.8×10^{-4}	126

FIGURE 5


NH R2
CH2
CH COO
R3
R1
NO2
EtO
EtO
POO
NO2
5.7
EtO
POO
CH2
NO2
1.5 x 10^4

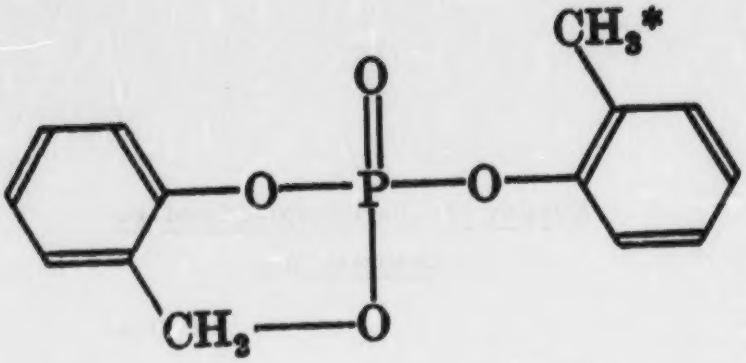
Hartley, Kilby (1950)
Becker et al. (1963)

FIGURE 6

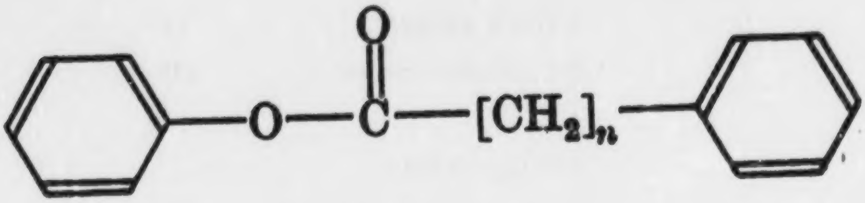


6×10^8
9×10^6
8×10^7

FIGURE 7


CH$_3$*
O
O
P
O
CH$_2$
O

(I)



(II)

FIGURE 8

Stability of dialkylphosphorylated true cholinesterase

	½ life (min)
Dimethyl	81
2-Chlorethyl	16
2-Chloropropyl	32
3-Chloropropyl	11
2:3 - dichloropropyl	139

Aldridge (1953)

Pickering, Malone (1966)

FIGURE 9

Important Factors in Acylating Inhibition of Esterases

Structure of inhibitor

Chemical reactivity of inhibitor

Type of esterase

Particular acyl group attached to esterase

Conditions of medium (pH, temp, ionic strength and composition etc.)

FIGURE 10

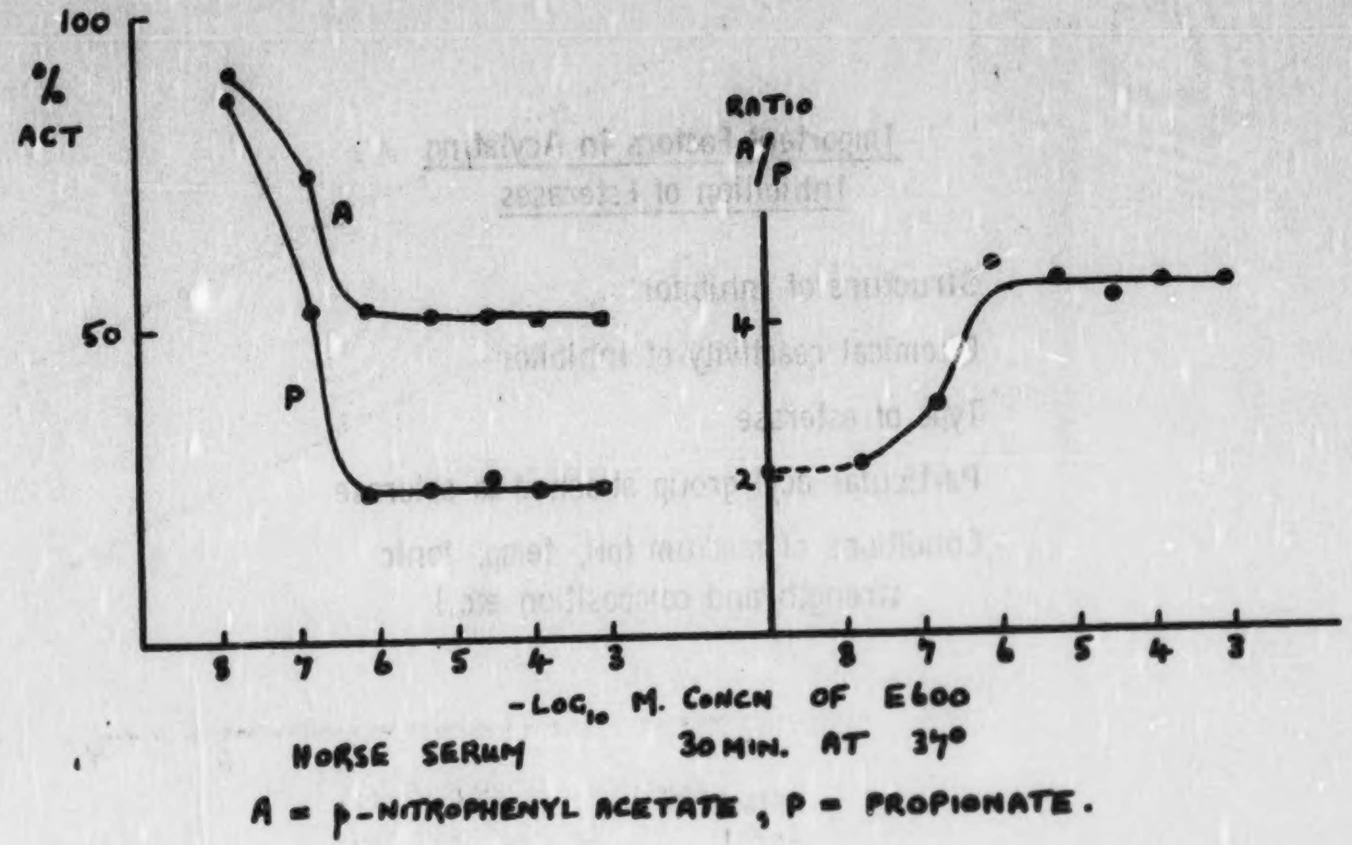

100
%
ACT
50
A
P
RATIO
A/P
4
2
8
7
6
5
4
3
8
7
6
5
4
3
-LOG10 M. CONCN OF E600
HORSE SERUM
30 MIN. AT 37°
A = p-NITROPHENYL ACETATE, P = PROPIONATE.

FIGURE 11

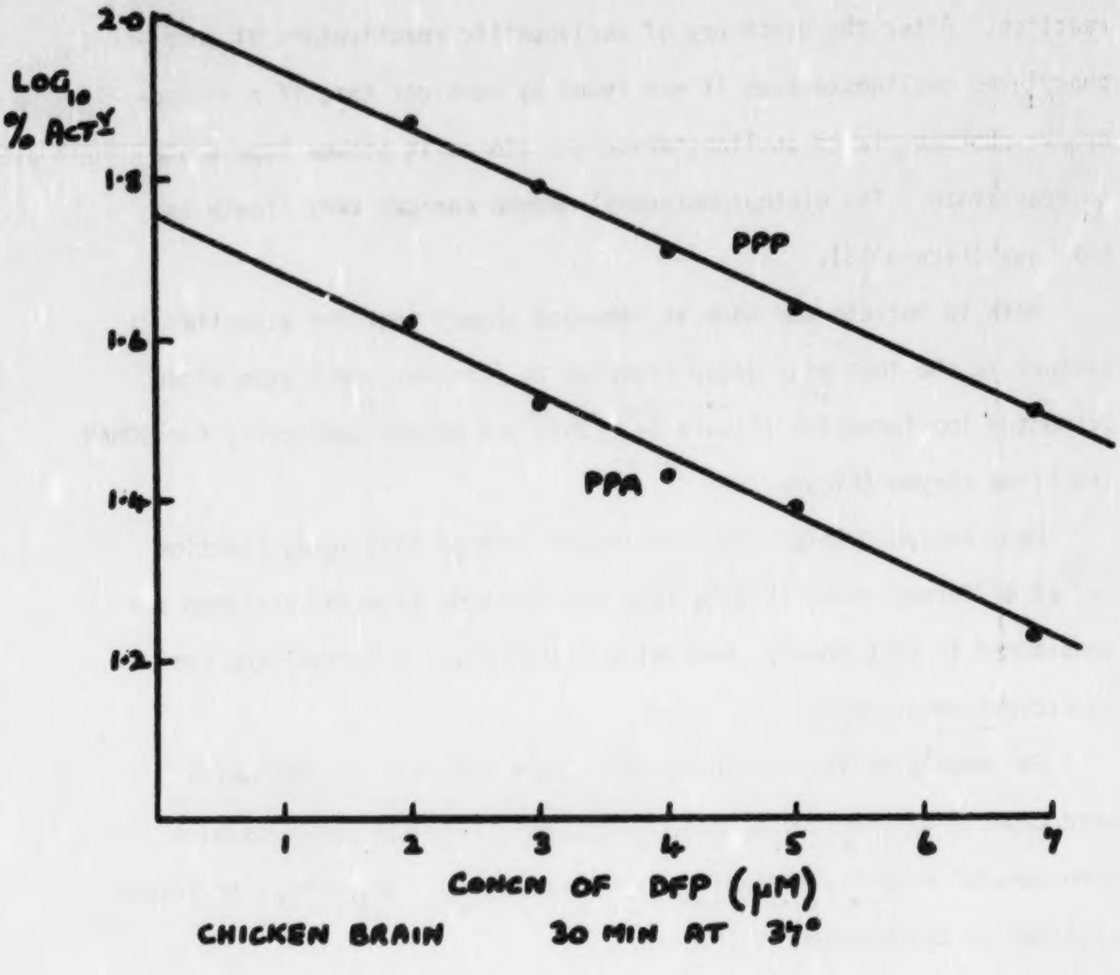

LOG10 % ACTY
2·0
1·8
1·6
1·4
1·2
PPP
PPA
1
2
3
4
5
6
7
CONCN OF DFP (μM)
CHICKEN BRAIN 30 MIN AT 37°

FIGURE 12

to be separated. Two examples are given - one a sensitive and insensitive enzyme, (Figure 11), and the other of two esterases of differing sensitivity (Figure 12).

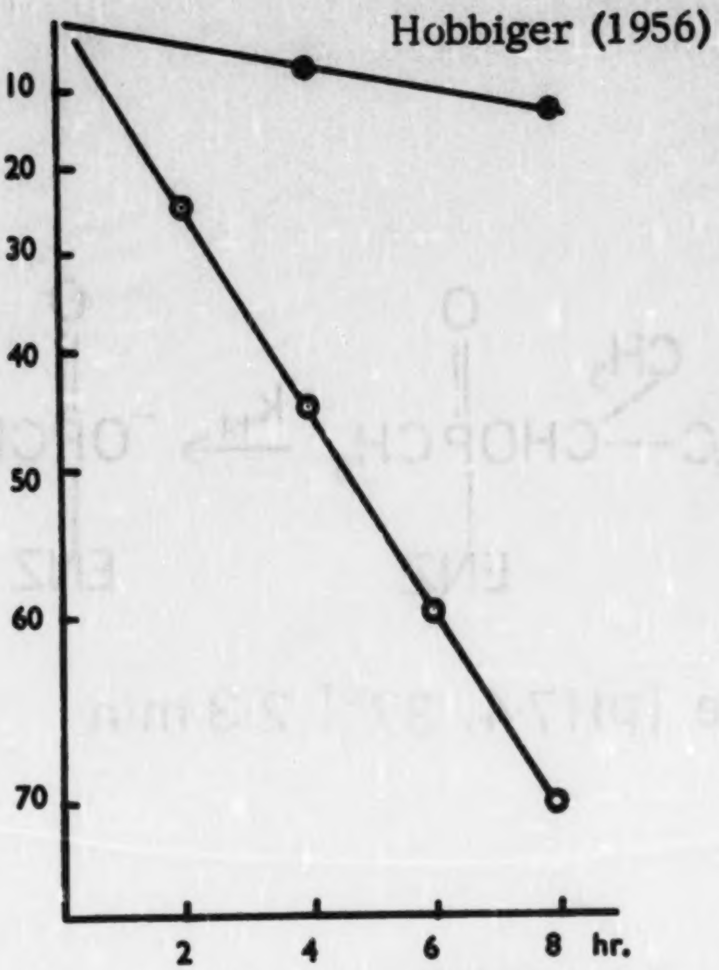
One reaction which has no substrate - enzyme analogy is the aging reaction. After the discovery of nucleophilic reactivators of phosphorylated cholinesterases it was found by Hobbiger that if a di-isopropyl phosphorylated cholinesterase was stored it became impossible to reactivate. The diethylphosphoryl enzyme changes very slowly in this way (Figure 13).

Work in Holland and here at Edgewood showed that the essential feature is the loss of a group attached to the phosphorus atom after carbonium ion formation (Figure 14); this was proved chemically for SOMAN inhibited enzyme (Figure 15).

Many phosphorylated cholinesterases undergo this aging reaction but at different rates (Figure 16); the reaction is acid catalysed and considered to be a purely chemical transformation not involving the participation of the active centre.

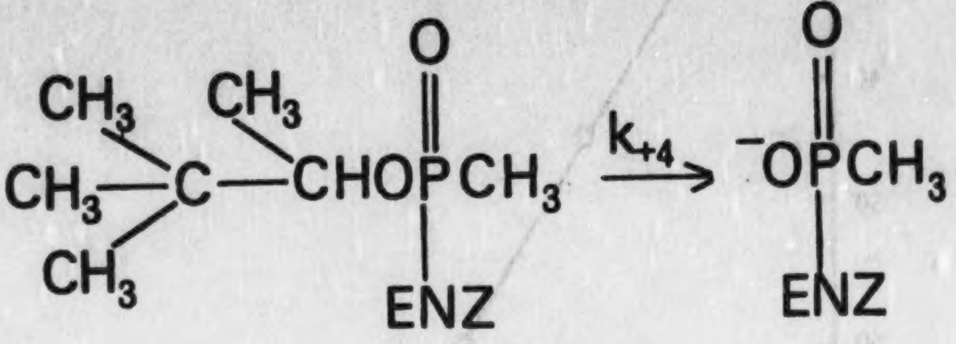
For some time the cholinesterases were the only enzymes which were capable of undergoing this change, but it is now known to be a more general property depending on the enzyme and the particular groups attached to the phosphorus (Figure 17).

The general view that the sole mechanism involves carbonium ion formation has now begun to look unlikely. For example, some straight chain alkyl derivatives were shown to age and Lee & Turnbull in 1958 had shown that diphenylphosphoryl chymotrypsin released one mole of phenol. The known difficulty of carbonium ion formation with phenyl



Enzyme reactivation after prolonged inhibition. Fresh human red cells were first incubated with the inhibitor. Small samples were taken after various periods of incubation and exposed for 1 hr. to 10^{-3}M-P_2AM. Enzyme reactivation was then determined after a 10-fold dilution. Similar results were obtained when high concentrations of AM were used for enzyme reactivation. ●——●: enzyme inhibited by an organophosphate containing a diethylphosphato group. ○——○: enzyme inhibited by an organophosphate containing a di*iso*propylphosphato group. Abscissa: duration of inhibition before addition of reactivator. Ordinate: % enzyme activity which cannot be restored by the reactivator (logarithmic scale).

FIGURE 13



½ life [pH7·4, 37°] 2·3 min

FIGURE 14

Rate of ageing of methylisopropoxy phosphorylated cholinesterase

Human erythrocyte, 37°

pH	10^3 x k(min^{-1})
6.0	10
7.1	4
7.2	3.8
8.0	3.0
8.5	1.2

Davies, Green (1956)

FIGURE 15

Phosphorylated enzymes which age

Acetylcholinesterase
Cholinesterase
Chymotrypsin
Carboxylesterase (Sheep, chicken, pig, horse)
Esterase on CNS membrane
("Neurotoxic esterase"; phenyl n-valerate)

FIGURE 16

pH 7.4, 37°

R_1	R_2	½ life (min)
$(CH_3)_2CHO$ -	$(CH_3)_2CHO$-	2140
CH_3-	$(CH_3)_2\ CH\ CH\ (CH_3)\ O$-	58
CH_3-	$(CH_3)_3\ C\ CH\ (CH_3)\ O$ -	6
$CH_3\ CH(Cl)\ CH_2O$-	$CH_3\ CH(Cl)\ CH_2\ O$-	160
$Cl\ CH_2\ CH_2\ CH_2O$ -	$Cl\ CH_2\ CH_2\ CH_2\ O$ -	32

COULT, MARSH, READ (1966)

PICKERING, MALONE (1966)

FIGURE 17

groups and the fact that the best substrates for chymotrypsin contain two aromatic rings suggests other possibilities.

Several examples are now known where aging and spontaneous reactivation occur at comparable rates. (Figure 18)

The pH dependence of spontaneous reactivation is bell shaped and for phenyl methylphosphoryl cholinesterases spontaneous reactivation and aging both have bell shaped pH dependence. This contrasts with the H+ catalysed reaction for branched chain alkyl derivatives. (Figures 19, 20 and 21)

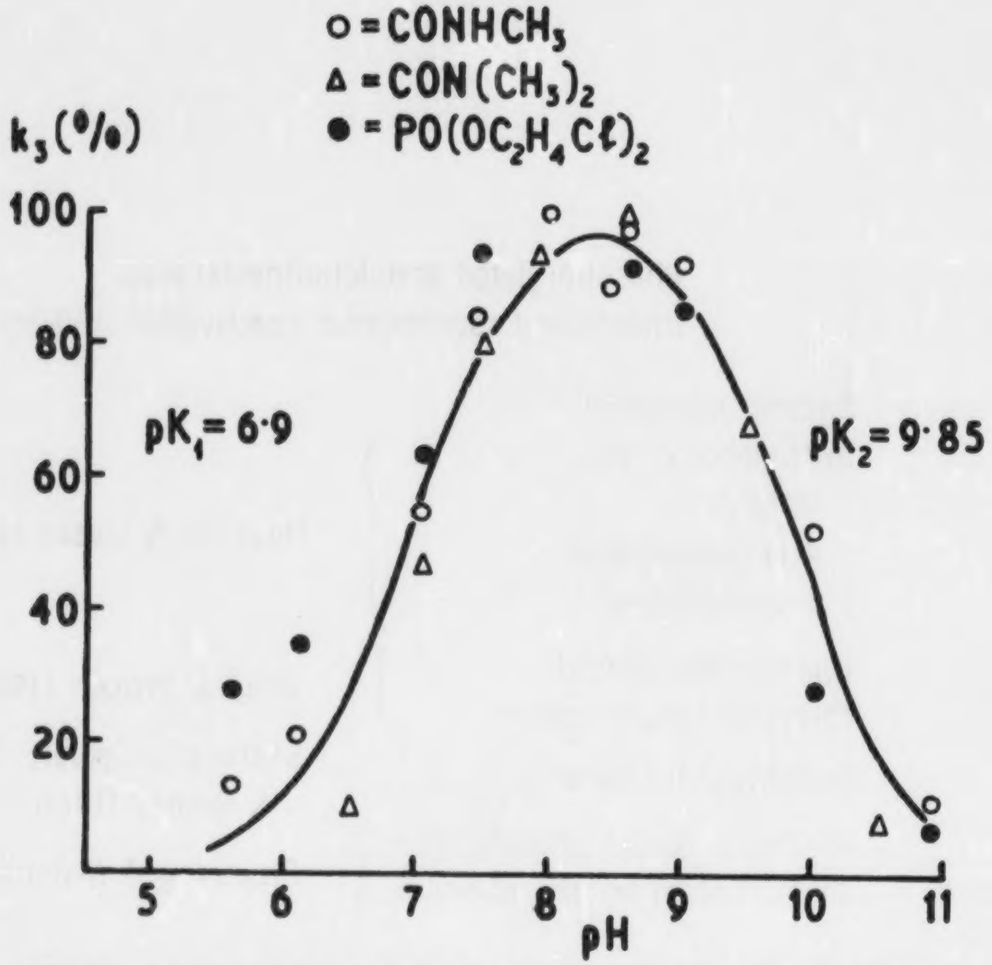
I therefore propose that aging can take place by two different mechanisms (Figure 22), one H+ catalysed through carbonium ion formation, and the other through a part of the normal enzyme reaction hydrolysing another ester bond. It will be clear from the structure-activity relationships for neurotoxic agents and the current hypothesis for the mechanism of delayed neurotoxicity necessitate this additional mechanism of aging.

This thumb-nail sketch of the reactions of organophosphorus compounds covers most of the background to the techniques and interpretation of results which will be developed in the next lecture.

Phosphorylated acetylcholinesterases undergoing spontaneous reactivation and aging

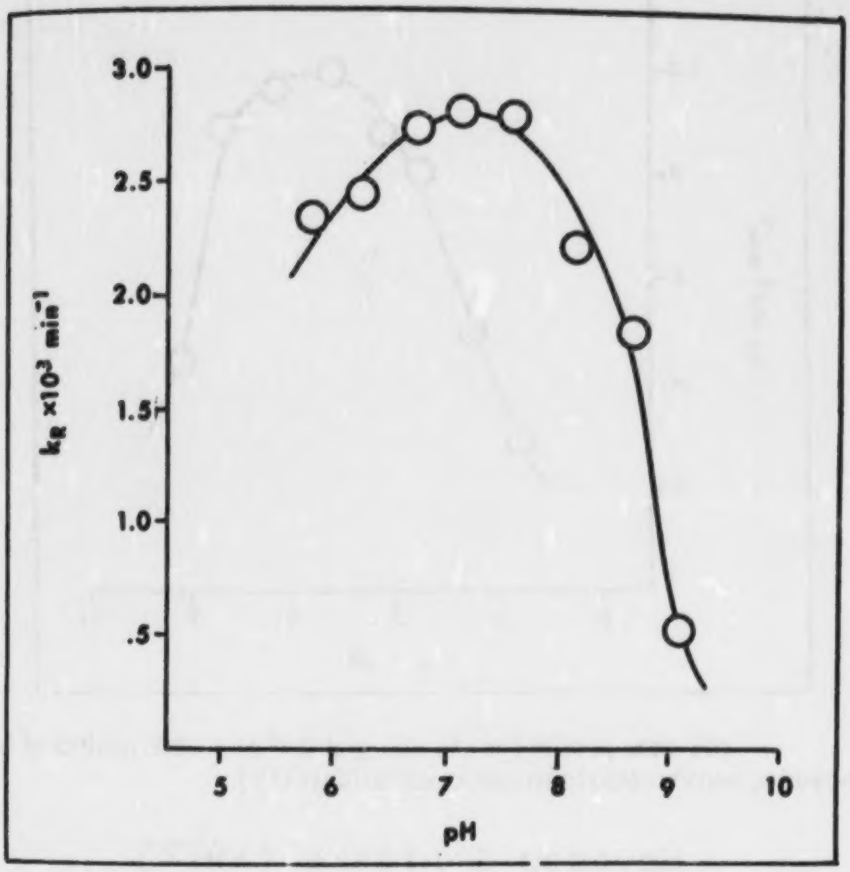
Enzyme derivative	
Methylphosphonyl -	Hovanec & Lieske (1972)
Phenyl	
4-methoxyphenyl	
4-cyanophenyl	
Diphenylphosphoryl-	Wins & Wilson (1974)
Phenylchlorophosphoryl -	
Dimethylphosphoryl -	Skrinjarić-Spolar, Simeon & Reiner (1973)
Di 2-chloropropylphosphoryl -	Pickering & Malone (1967)
Di 3-chloropropylphosphoryl -	

FIGURE 18



RATE CONSTANTS OF REACTIVATION OF INHIBITED ChE. THEORETICAL CURVE CALCULATED FROM OBSERVED pK VALUES

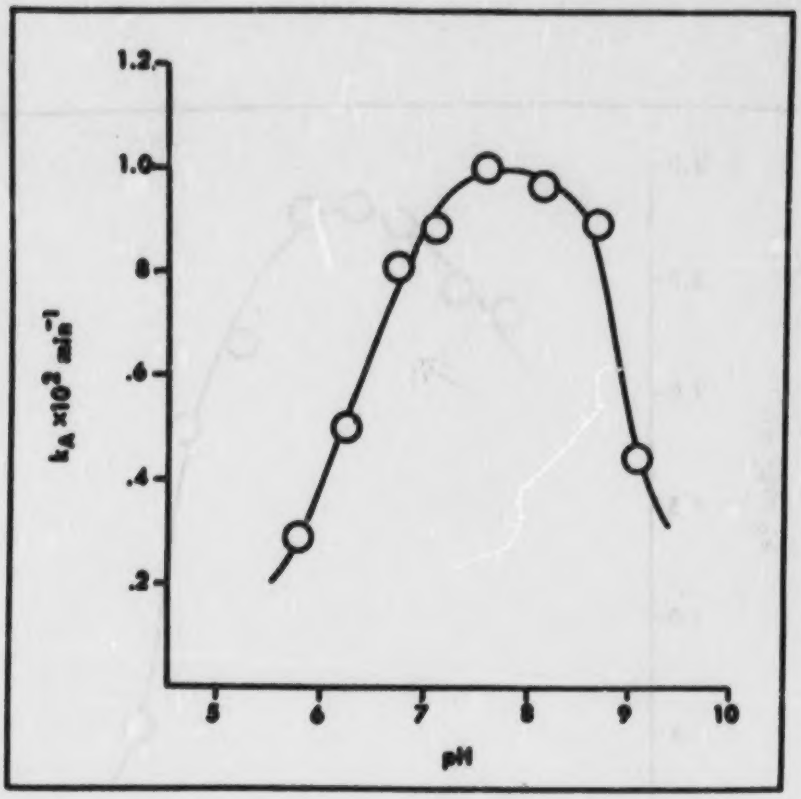
FIGURE 19



pH–rate profile for the spontaneous reactivation at 25° of AChE inhibited with *p*-cyanophenyl methylphosphonochloridate (IV).

Hovanec and Lieske (1972)

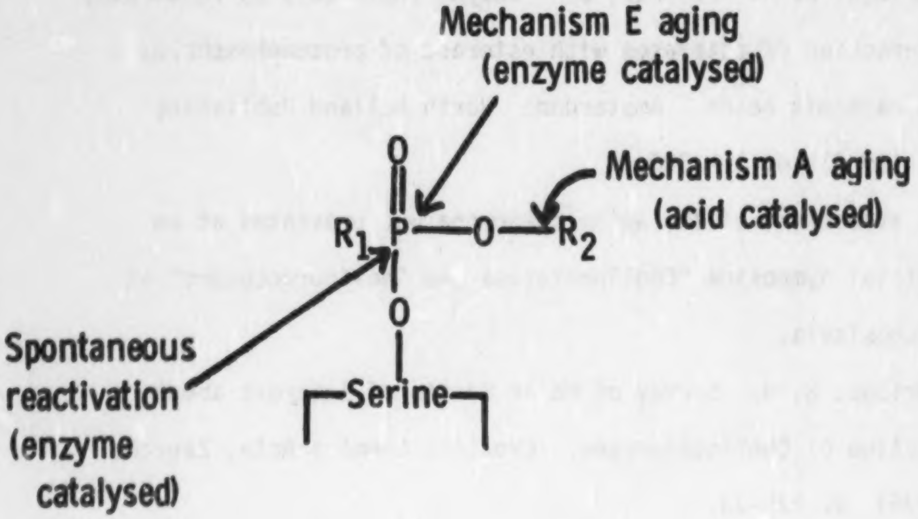
FIGURE 20



pH–rate profile for the aging at 25° of AChE inhibited by *p*-cyanophenyl methylphosphonochloridate (IV).

Hovanec & Lieske (1972)

FIGURE 21


Mechanism E aging
(enzyme catalysed)
Mechanism A aging
(acid catalysed)
O
R_1
P
O
R_2
O
Serine
Spontaneous
reactivation
(enzyme
catalysed)

Reactions of phosphorylated cholinesterases

FIGURE 22

References

A detailed discussion of the reaction of organophosphorus compounds and esterases has been published.

Aldridge, W. N. & Reiner, E. 'Enzyme Inhibitors as Substrates' interaction of esterases with esterase of organophosphorus and carbamic acids. Amsterdam: North Holland Publishing Co. (1975) p. 1 - 328.

A recent assessment of the aging phenomena was presented at an International Symposium "Cholinesterase and Cholinoreceptors" at Split, Yugoslavia.

Aldridge, W. N. Survey of Major Points of Interest about Reaction of Cholinesterases. Croatica Chemica Acta, Zagreb, (1975) p. 225-33.

THE MECHANISM OF ACTION OF NEUROTOXIC ORGANOPHOSPHORUS ESTERS

M. K. Johnson, Ph.D.

Professor Casida, ladies and gentlemen. I think it right that we acknowledge that the ability to get an understanding comes from almight God. It's also very true that every scientific worker climbs upon the shoulders of every other worker. In some cases other workers grab him by the legs and try to pull him down, but in others they seize him by the ankles and hoist him up! I'm fortunate in that I've been in the situation of the latter at Carshalton. We have already heard the tribute to the late Dr. John Barnes. Both he and Dr. Aldridge have consistently and over many years hoisted me up in order to help on the investigations which they set in motion.

I want to say three negative things to start with. Firstly, to reemphasize the fact that we really ought not to talk or think in terms of demyelination. It's true that if you wait long enough after a long and severe assault, you will see myelin degeneration. But the primary degeneration, as Professor Casida has already pointed out, is undoubtedly degeneration of axons. And "demyelination" only serves to deflect both the pathologist and the biochemist from looking for the right sort of lesion.

Secondly, to remind us that not all anticholinesterases are inevitably neurotoxic. And thirdly, to say that we're not speaking about the effect which has been described as an immediate paralytic effect of one or two pesticides, by Durham, Gaines, and Hayes (1956),[1] and Witter and Gaines (1963).[2] I believe there is evidence in the original papers which can be interpreted to show that that is a prolonged, chronic cholinergic effect. So we're talking about what I would like to call delayed neuropathy.

Organophosphorus compounds which cause delayed neurotoxic effects phosphorylate a characteristic nervous-tissue protein,[3] and inhibit its activity as an esterase.[4] There is ample evidence that the phosphorylation measured radiochemically and the inhibition measured enzymatically both represent the covalent reaction of the inhibitor at a single enzyme active site. It is important to the understanding that we are not beguiled into proposing a second site to explain the results with protective agents (see below).

In vivo studies with hens using a variety of inhibitors of neurotoxic esterase reveal that the toxic effect does not occur directly because of the loss of esterase activity. The toxic response depends on the chemistry of the inhibited enzyme.[5] Two classes of inhibitors of neurotoxic esterase have been defined (Figure 1). Class A compounds (phosphates, phosphonates and phosphoramidates) are neurotoxic. Class B compounds (phosphinates, sulphonyl fluorides and carbamates) are not only non-neurotoxic but actually protect hens against subsequent challenge doses of Class A compounds.[5,6,7] It is probable that a process analogous to the "aging" of inhibited cholinesterase occurs after neurotoxic esterase has been inhibited by neurotoxic (Class A) agents. The charged acidic group which then would be left attached to the membrane-bound neurotoxic esterase could disrupt normal metabolism in the neurone. Speculations

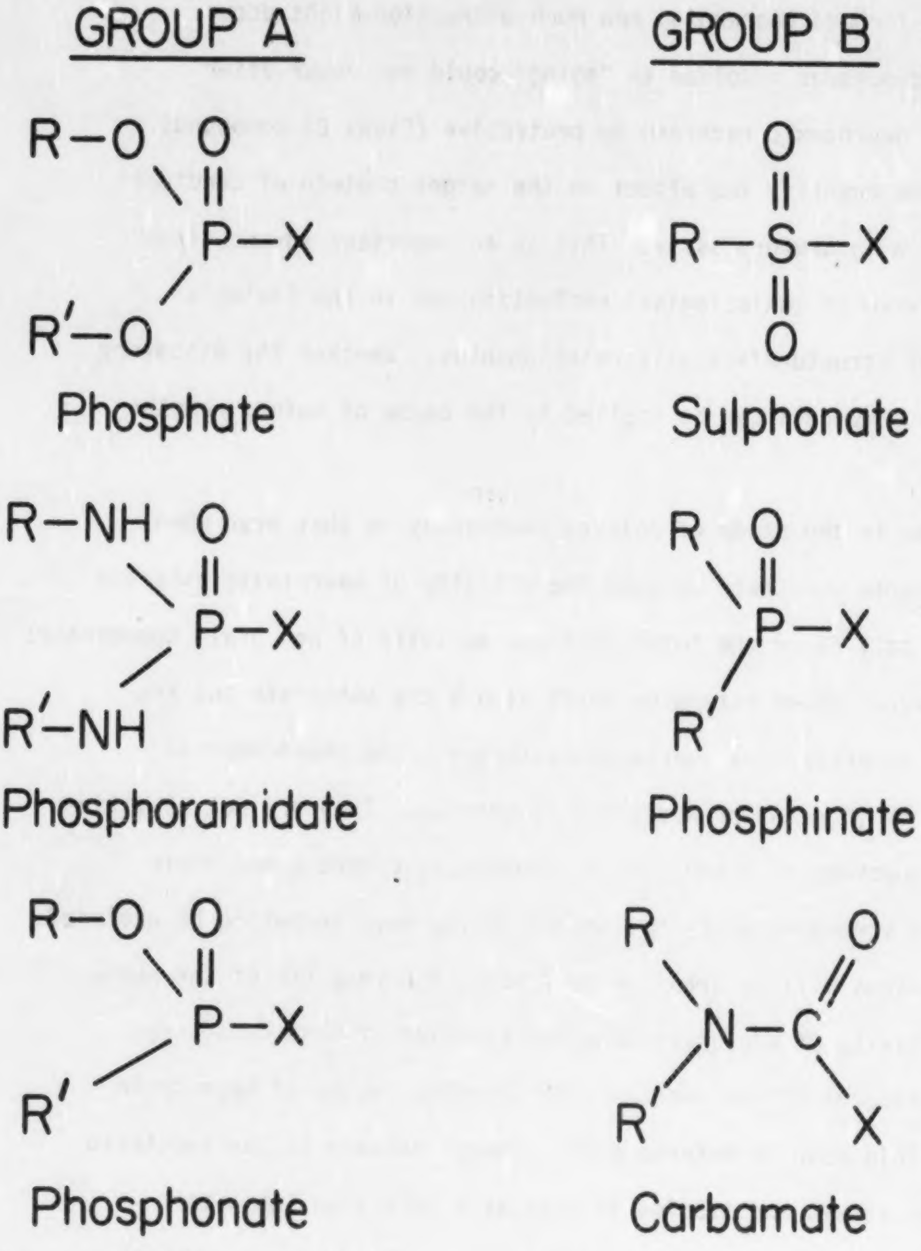

GROUP A
GROUP B
Phosphate
Sulphonate
Phosphoramidate
Phosphinate
Phosphonate
Carbamate

FIGURE 1. Compounds which belong in Group A and which are found to inhibit neurotoxic esterase in vivo are neurotoxic. By contrast those of Group B which will inhibit NTE in vivo are protective.

have been put forward concerning how much disruption might occur.[8] The chemical processes involved in "aging" could not occur after inhibition of neurotoxic esterase by protective (Class B) compounds.

We can now quantify the effect on the target protein of candidate pesticides by a laboratory assay. This is an important advance from the point of view of toxicological evaluation and in the fields of therapy and of structure/activity relationships. Whether the discovery of protective compounds can be applied to the cause of safety remains to be seen.

A problem in the study of delayed neuropathy is that even when the most favoured substrate is used the activity of neurotoxic esterase accounts for only 6% of the total esterase activity of hen brain homogenate: there are several other esterases which attack the substrate but are distinct and separate from neurotoxic esterase. The physiological significance of these other esterases is unknown. They are not sensitive to the same spectrum of inhibitors as neurotoxic esterase and their presence in a screening assay for neurotoxicity must therefore be excluded. False conclusions will be drawn by monitoring the response of the whole esteratic activity of hen brain to administration of test compounds. A detailed protocol of the necessary differential assay of neurotoxic esterase is laid down in Reference[12]. Phenyl valeate is the substrate of choice for assays and mipafox is used as a selective inhibitor to pick out neurotoxic esterase from irrelevant (paraoxon-sensitive) esterases which also attack this substrate. Phenyl benzylcarbamate is a safer but not completely selective alternative to mipafox. A better alternative is needed and I hope to submit a report soon to Toxicology and Applied Pharmacology describing a safer and simpler assay which will be suitable for screening of dosed hens: it is not suitable however for checking active compounds in vitro.

The relationship of structure to activity in vivo has been explored for a wide range of compounds.[9,10,11] These are related both to potential pesticides and also to the plasticiser-type tri-aryl phosphates. It has been possible to indicate certain features of structure which tend to be associated with low neurotoxic potential and some generalisations are summarized in the next paper. The respone of single hens to test doses of compounds can be assessed biochemically one day after dosing. The effects of doses below those causing frank ataxia can be graded in a fashion not possible in the all-or-none clinical test.

Investigations of the mechanism of action of neurotoxic organophosphorus esters have been undertaken over a period of more than 40 years. A number of blind alleys have been explored, but it is certain that the more recent successes would not have been achieved without the earlier work. The way in which these fundamental studies would prove relevant to toxicity testing and to safety could not be foretold. Enzyme processes have been scrutinized in great detail, and studies have not been restricted by the demands or criteria of short-term projects. It is only recently that the practical benefits to toxicology have begun to be appreciated. However the benefits are considerable - coherent structure/activity studies have been performed with a suitable monitor; prospective forecasts of activity are possible; chronic dosing can be evaluated; preventive measures are possible; and therapy is being explored rationally; species variation and human sensitivity can be explored more clearly. Many further practical investigations utilizing present understanding have been proposed.[12] This does not mean that mechanistic studies should cease. Rather, efforts should be increased in the confidence that further understanding of mechanism will be valuable, and possibly in unforeseen ways. Further work is needed to

(a) confirm, if possible, the "aging" hypothesis;

(b) locate neurotoxic esterase intracellularly;

(c) purify and study the properties of this curious protein in hens, man, and other species;

(d) discover its physiological function as distinct from its apparently unnecessary esterase activity;

(e) elucidate the sequence of events between phosphorylation of neurotoxic esterase and axonal degeneration;

(f) study the processes at work in maintaining connection of nerve endings with their receptor organs;

(g) try to find links between neuron degeneration caused by organophosphorus compounds and that caused by other chemicals and disease agents. In spite of morphological similarities in some cases, there is no evidence as yet that these neuropathies share a common mechanism.

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Discussion

DR. J. ALBERT: We've talked for some time about species differences. Have you looked at the rat and the chicken with respect to the enzyme inhibitors?

DR. M. JOHNSON: Yes, I have looked. And quite clearly, the species difference doesn't reside in this preliminary step. Rats are not sensitive but have an inhibitable neurotoxic esterase as far as simple comparative characterization goes. Baboons and sheep both have the enzyme. Sheep are susceptible and the neurotoxic esterase is inhibited by the appropriate neurotoxic dose of haloxon. In young baboons, the enzyme is inhibited by the saligenin cyclic phosphates, but I've never had an adult baboon for a clinical test: it was very young and so the testing there wasn't really strictly relevant.

Young chickens, are also resistant apparently, but if you give multiple doses, you can clearly get ataxic chickens. However, the first dose was adequate for full inhibition. So it looked as though there was some sort of an adaptive process. Perhaps in growing birds it's easier to explain than actual differences between species. In the growing bird, perhaps other axons take over and the bird adapts as it grows.

For the difference of species, guinea pigs are resistant to a single dose as Professor Casida pointed out. However, there is evidence that if several doses are given, you can get an ataxic guinea pig. It is possible that the difference lies in differences in rate of ageing but I wouldn't hang my hat on that. I think it's a difference in sheer anatomy, physiology. Maybe size of axons. It seems to be the smaller animals which are the least susceptible.

For monkeys and some other primates, I don't think the testing has been fair. Because it's really only been done with TOCP. My conviction is that if you're really going to check something, you ought to use a direct inhibitor rather than one which needs metabolic activation and may also be poorly absorbed. You should use the cresyl or the phenyl saligenin phosphate, if you really want to know about TOCP.

So the answer about species is we don't know and I'm making guesses.

DR. N ALDRIDGE: Could I just re-emphasize, without underestimating the difficulty with the rat, that over the last five to ten years, I think there has been a reorientation in our view. Ten years ago it was thought that the chicken, cat and man were the only sensitive species. There is now quite a catalogue of sensitive species, particularly, as Dr. Johnson said, the larger species. Sheep, cows, horses, pigs, and water buffalo, are all very sensitive. We are beginning to realize that these compounds are attacking some fundamental property of the central nervous system. We now should look to why the mouse and the rat are exceptional.

DR. C. C. LEE: A comment on Professor Casida's remarks concerning the rats and the dogs, and their being non-reactive to neurotoxic agents. Three years ago we undertook a long-term evaluation of the di-methyl, di-thio carbamates, sponsored by NIEHS.* After feeding rats high doses of Ferbam or Thiram, commonly used fungicides in the United States and overseas, for up to 18 months, a neurotoxicity developed, characterized by ataxia, Parrota's a paralysis of the posterior part of the body, degeneration of myelin sheath, and axon degeneration.

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* National Institute of Environmental Health Sciences, Dept. HEW, Research Triangle Park, North Carolina

The animals, before developing the neurotoxic effect displayed, a series of abnormal behavioral activity in tests, which might indicate the subtle changes due to a neurotoxic effect.

DR. J. E. CASIDA: Could you restate the compound?

DR. C. C. LEE: A dimethyl-di-thiocarbamate. The compound is ferbam, which is the ferric salt. This type of compound was developed because interest in the rubber industry. It was subsequently discovered that these molecules have medical uses and also as pesticides.

Antabuse, which is used in the treatment of alcoholism, belongs to this group. Detailed information will be presented to a symposium March 1st, 2nd, 3rd, 1976 at Research Triangle Park, N. C., sponsored by WHO and NIEHS, concerning the chemicals of interest to the rubber industry. The proceedings will be published in Environmental Health Perspectives.

DR. N. ALDRIDGE: I think we must be absolutely clear that what is being proposed by Dr. Johnson is the mechanism of the production of a dying back lesion in the peripheral and central nervous system by organophosphorus compounds. It is clear that other compounds will produce dying back lesions in the peripheral nervous system by mechanisms which are not the same. For example, acrylamide produces a beautiful dying-back lesion in rats and other species which does not involve neurotoxic esterase. As for the carbamates that have just been mentioned, these compounds do not carbamylate, so they are compounds of a quite different nature. There are probably many different mechanisms of producing a dying back lesion in the central nervous system.

DR. T. EDWARDS: I would like to ask about reversibility of the harm from neurotoxic organophosphates. I was wondering if you've done any work on the reversibility of this dying back effect, or whether it's some other further complication that's caused persistence of the lesion.

DR. M. JOHNSON: I think it's well known that in adults, both men and hens, that once a severe ataxia comes on from organophosphorus esters, then there appears to be really no improvement in clinical state. This is different from the acrylamide neuropathy, for instance, which Dr. Aldridge has just referred to.

In terms of the enzyme, we need to be clear that the initial biochemical event, can be induced within one hour of dosing. The earliest pathological, microscopic lesion is only seen after 6-8 days and at that time resynthesis of enzyme has caused a significant return of activity as you saw on one of the slides. I want to emphasize and perhaps keep on emphasizing that it isn't the loss of enzyme activity that matters. We have insulted the neuron in some way, as from one hour after dosing, that it doesn't like. And at the moment there doesn't seem to be any way of reversing that biochemical insult. And from then on, everything progresses. It seems to me rational that, with the understanding that we now have, it may be possible to construct reactivating agents analogous to PAM, but appropriate to the active site of neurotoxic esterase. In fact I've listed some in that review in Critical Reviews in Toxicology, which seemed to me appropriate. And we are engaged actively in looking at some of these. So far, I would say, with only moderate success whether, even if we did reverse the inhibition and pull that charged group off the enzyme, whether there would be no onset of frank lesions remains to be seen.

DR. N. ALDRIDGE: I don't know whether there is a neuropathologist who would comment on the well known fact that neuropathy in the peripheral nerves recovers completely, whereas in the central nervous system, if it once progresses to a disconnection, there appears to be no recovery.

DR. T. EDWARDS: I don't have an explanation, but I have run across some references where the difference in species would depend on the ratio between sphingomyelin and lecithin in the nerve. And of course there would be a difference between peripheral and brain as well as a difference in peripheral nerves in various species.

DR. M. JOHNSON: I have discussed that idea of Rosenberg's in my critical review: it is interesting but some data may not be too good.

DR. J. SANBORN: You commented on the fact that young chickens aren't as susceptible to neurotoxic agents, except by multiple dosing. Have you tried to characterize this enzyme in young chickens as opposed to chickens that are older and does any of this have anything to do with the chickens when they become old and are in full egg production? Is there any kind of steroidal implication between the egg production and the development of this esterase? The systems are, of course, very different physiologically, but I'm curious about this.

DR. M. JOHNSON: Well, to answer the second part first, the same age dependence is seen in man. Children in Morocco, for instance, were far less severely affected, and they tended to improve in time. I think in Canada there has been a publication in TAP* awhile ago on the contrast between adult cats and young kittens with one of the saligenin phosphates. And so I don't feel it's so much perhaps developmental in that sense, but perhaps the nervous system is still developing and the axons are certainly growing. In my simplistic view, I think that's more significant.

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* Toxicology and Applied Pharmacology.

As regards the enzyme, the obvious characteristics in chicks and adult hens appear to be identical. And indeed, I think it would be hard to think of it otherwise because the chick goes through a gradation where it begins to become somewhat sensitive and then fully sensitive. And I find it hard to believe that the enzyme is changing in a graded way. Also the half life of inhibition and return is just the same in the young chick as the adult chick.

DR. J. DOULL: I'd agree with Dr. Johnson's comment on children. There's another phenomenon there, and that's plasticity that occurs in children, whereby an axon can switch from one cell body to another. My question has to deal with therapy. I had hoped that you were going to offer us some hope with the statements that you would make about the sulphonates and the phosphinates and so on. But I thought I understood you to say that when they were given after exposure, they were worthless. Is that correct?

DR. M. JOHNSON: That is absolutely right. They are perfectly effective as protective agents. But once the appropriate covalent bond has been formed at the enzyme, there is nothing that another acylating agent can do. You need a quite different class of compounds, such as oximes tailored to this enzyme. Whether we shall ever find one, remains to be seen. I live in some hopes.

DR. J. DOULL: Let me rephrase my question. Do you have any thoughts or recommendations that would be useful in the way of therapy to modify that lesion before the axonal degeneration really gets set? If we're saying, that there is a period there in which we could intervene to prevent degeneration starting from the end of the axon, how would we go about that?

DR. M. JOHNSON: I have absolutely no suggestion.

DR. N. ALDRIDGE: For reactivation of phosphorylated cholinesterase, the oximes are tailored to utilize the active center, i.e., they have positive charges. They are however totally ineffective when the enzyme is aged. No method has been found to reactivate aged enzyme and it would clearly require something quite different from an oxime.

DR. S. FRAZIER: What is the relationship with the changes in the cholinesterase of the brain, serum and so forth, in determining neurotoxicity?

DR. M. JOHNSON: There is no correlation whatsoever. We are dealing with two separate enzymes, each with its own structure/activity relationships which can be characterized as you would characterize structure activity for any other substrate or inhibitor or any other enzyme. There is, however, of course, some overlap, so that some compounds which have some anticholinesterase activity also have some anti-neurotoxic esterase activity. But perhaps when we come to talk about structure activity a little more, I can point out some of the chemical features which favor either potent inhibition of neurotoxic esterase, or possibly discourage it. You can run the whole gamut with OP compounds on the one hand you can inject a compound which will produce negligible cholinergic response and yet cause delayed neuropathy. On the other hand there are many compounds which only show the delayed neuropathy if you give really quite heroic treatments to prevent them succumbing first to the acute anticholinesterase effects.

DR. G. KIMMERLE: If you are dosing hens, orally or by other routes of administration, and you give your animals different dosages, a range from very low dosages to high dosages, and protect them with oximes, do you see a dose relationships in your enzyme bio-assay? In using compounds which are neurotoxic to chickens in doses from 1 to 1000 mg/kg, can you find a dose relationship in your bioassay?

DR. M. JOHNSON: Yes, I can find a dose relationship. You will find some of the data in that review in Archives of Toxicology. In the Tables there are a number of compounds which have been tested at various dosages. And you will see a column which says, "Percentage Activity Remaining of NTE". When you see 70% or 50% remaining, the compound is not neurotoxic at that dose but when you find residual activity around 20% or less, then you'll always find a positive neurotoxic response marked off in the next column. There is quite a clear dose-response relationship.

DR. N. ALDRIDGE: I don't know whether the questioner was hinting at another question which really has not been fully explored. As Dr. Johnson says, his data indicates that at a certain level of inhibition you obtain a clinical response in the hen. There is another question which can be raised, which, perhaps later on in the meeting will be explored a bit more;

whether the meaning of percentage inhibition is a statistical statement. Does it mean if you have a 70% inhibition in one neuron that you have 100% in some and 30% in others. These are all sorts of quantitative questions which maybe one can begin to answer. The ability to make quantitative measurements on the receptor will greatly aid movement forward in answering such questions.

DR. V. K. H. BROWN: I was very perturbed, sir, to see in your early slide that you listed dichlorovos as being neurotoxic. I was wondering whether any of the speakers could tell us the level of evidence for this. As I understand it, from the work of Albert and the work of Dr. Johnson, the long chain analogues, C5 longer chain analogues of dichlorvos may be highly neurotoxic. But I understood that the dimethoxy compound was not a cause of delayed peripheral neurotoxicity. Could I have a comment on this, please?

DR. M. JOHNSON: I did react, perhaps in a slightly modified version to yourself, seeing that on the slide. And in the sense that it is possible under the most heroic conditions imaginable, making the hen house look like a surgical operating theater and dosing with dichlorovos on two successive days, at 100 milligrams per kilo on each occasion (each about 10 X unprotected LD_{50}) with pre-dosing with eserine and atropine plus oxime, you can just about get them through the acute effects and show a paralytic effect. But this is very different from the other five compounds which were listed on Professor Casida's slide: they all produce a neurotoxic response with a far greater degree of ease in conditions where acute cholinergic effects were much less severe. Now, I think we must allow the fact that, in a research laboratory, I have been trying to squeeze the last bit of information out of structure/activity relationships, and have therefore gone to quite unprecedented lengths, in order to keep a bird alive. I found that, at a lower dose, dichlorvos did produce some inhibition, and I wanted to know what would happen if I could force the inhibition up. But I think you are fully justified in pointing out that dichlorvos is really in a very different class from EPN or leptophos, for instance.

DR. J. ALBERT: I would agree with Dr. Johnson that with heroic efforts we can perhaps keep these birds going and obtain paralysis. I would like to know if there was a distinct recovery phase on those birds before they showed the paralysis and the degree of paralysis that you did see after that period of time.

DR. M. JOHNSON: There was a distinct recovery phase. They had recovered sufficiently within 24 hours after the first dose to be able to survive a second identical dose on the second day and they were quite o.k. two days later. May I shift my ground a little bit to another dimethyl compound? With dimethylphosphorofluoridate, you can give a dose such that they look almost dead within about three minutes of dosing. Ten minutes later they're picking up their heads, and half an hour later they're walking around the cage. With that compound also, it's necessary to give two heroic doses. I think we could say that those two are the only dimethyl compounds which are likely to have structures with much fit for the enzyme. There is a clear recovery stage for both these compounds and the hens walk around healthily for days before they become paralyzed.

DR. J. ALBERT: I have just one short technical question on the phosphinates. Do you see inhibition of only the neurotoxic esterase, or do you see also a paraoxon-like inhibition and an additional inhibition beyond this.

DR. M. JOHNSON: Every one of the OP compounds I've tested has got its own spectrum of specificity. A number of them are quite good inhibitors of the paraoxon-sensitive enzymes, but I haven't always checked those. I have gone straight to what I call the B minus C assay, so I can tell you something about the effect on neurotoxic esterase and on the residual seven percent. I don't know about phosphinates on the paraoxon-sensitive enzymes.

STRUCTURE/ACTIVITY RELATIONSHIPS AMONG ORGANOPHOSPHORUS ESTERS WITH RESPECT TO DELAYED NEUROPATHY

M. K. Johnson, Ph.D.

In the Mechanism talk (see previously) I explained that the primary target in organophosphate neuropathy is the esterase site on a nervous system protein which we call "neurotoxic esterase." It is possible to correlate neurotoxic doses with high inhibition of neurotoxic esterase assayed in homogenates of whole hen brain. It is clear that we are using the brain (which is easily removable from a carcass) as a monitor for the effect in spinal cord and peripheral nerve; lesions are most marked in these latter tissues but the tissues are less convenient for routine biochemical investigations. The validity of this practice of monitoring brain is proven by the results. I have published recently an extensive review of structure/activity relationships[1] and my purpose here is to state a few of the major generalisations which have emerged. For compounds which are directly active against neurotoxic esterase in vitro it has been possible to understand the relationships more easily since we are not confused there by problems of distribution and differences in activation and disposal mechanisms. I have reported[2] on the relationships in vitro for organophosphorus esters and also for carbamates and other compounds which might be useful as protective agents or as analytical tools.

On structure/activity, this meeting is, of course, committed to interest in pesticides. But we've seen quite clearly that the mechanism is common to both the pesticidal organophosphorus esters and to the tri-aryl phosphates which have been responsible for the majority of the human cases of delayed neuropathy. And since there are some quite pretty structure/activity relationships there, I thought it would be instructive just to look at them briefly, before we go on to the pesticidal compounds. The classical neurotoxic compound, tri-_ortho_cresyl phosphate, has been studied by many workers. But when we talk about tri-_ortho_cresyl phosphate, I think we ought to be perfectly clear that probably 99.9% of all the work which has ever been done with tri-_ortho_cresyl phosphate has not, in fact, been done with pure compound. To get pure _ortho_cresol in former days from coal tar distillates was not a practical proposition. One got preparations which were labelled "_Ortho_cresol", "_Meta_cresol", "_Para_cresol", but they were all crude mixes, containing varying percentages of the isomers plus some phenol or perhaps a substituted ethyl-phenol. Now, this is very important indeed, because there are vast variations in activity of the triesters according to the variants of the substituents on the three aryl groups. This has been thoroughly documented by three different groups of people. First of all, Henschler in Germany has done a colossal amount of work. And then also Bondy, Field, Worden and Hughes, and also Dr. Barnes and Dr. Aldridge had a look at substituents, in collaboration with Bondy of the Coalite Chemical Company in Britain. It is now quite clear that if you compare the symmetrical tri-esters having ortho substituents with esters which have only one ortho-substituent, plus two, say, phenyl groups, or two _para_-cresyl groups, you will get a compound which is much more neurotoxic. This means that if you start with a feedstuff which you thought was pure _ortho_cresol and make tri-ester with phosphorus oxychloride, you won't, in fact, get a hundred percent tri-orthocresyl phosphate. You'll get maybe 97 or 98% pure tri-ester and you'll also get varying amounts of the dicresyl mono-phenyl ester, and diphenyl mono-orthocresyl ester. So if you start with orthocresol contaminated with phenol, you'll get a much more neurotoxic compound. And therefore when we talk about comparing man and hen, as regards their sensitivity to triorthocresyl phosphate, we are on a very sticky wicket. It cannot really be done. Nobody knows what was the actual chemical entity in ginger jake or in the Moroccan cooking oil. Certainly tri-orthocresyl phosphate was a substantial proportion of it. But it is also almost certain that there were a number of other isomers of varying degrees of activity, and in many cases much higher activity. I say that, by the by, because obviously this business of species comparisons is important to us. So, having said all that, let's go back to structure/activity.

The first obvious contrast which came along was that the ortho compound was very neurotoxic, while the tri-paracresyl compound was not neurotoxic, even on repeated doses. This is supported by the biochemical test which shows high and negligible inhibition respectively one day after dosing. However, when testers looked at the ethyl substituents, the situation was reversed. The tri-orthoethyl compound was not neurotoxic and the tri-paraethyl compound was neurotoxic! And there it was! There was an impasse on the "score zero" versus "score one" test. The value of the biochemical test in this situation is clearly seen in Table 1 which compares both clinical and biochemical test data for the isomeric tri-ethylphenyl phosphates. Biochemical testing confirmed the neurotoxic potency of the para isomer but it showed that the ortho isomer was by no

ISOMERIC SYMMETRICAL TRI-ETHYLPHENYL PHOSPHATES

Position of Substituent	Dose (mg/Kg)	Inhibition (%) of NTE	Ataxia seen
Para	250	92	+
Ortho	1200	42	-
	4 x 1200	87	+
Meta	1200	4, 9, 16	-
	9 x 1200	4	
	11 x 1200	-	-

Table 1 Results of biochemical and clinical monitoring of three isomeric tri-aryl phosphates. Inhibition of neurotoxic esterase was measured 18-24 hours after the final dose and clinical assessment was made daily until 28 days after dosing.

means inactive (42% inhibition of neurotoxic esterase after a single dose). Subsequent testing of 4 doses showed the parallel result of high inhibition and clinical ataxia. In this case biochemical monitoring revealed and predicted a hazard. In the case of the meta isomer the test revealed negligible biochemical response after a single dose and the Table shows that the consequent prediction that multiple doses of this compound would also be ineffective has been confirmed. Thus the brief biochemical test after a single dose has effectively distinguished between two compounds as being hazardous and safe respectively, whereas clinical testing alone did not reveal the neurotoxic potential of the one compound and could never quantify the margin of safety for the other.

Now, it's true to say that tri-aryl phosphates are intended to be inert chemicals. They are synthesized as plasticizers, hydraulic fluids or flame retardants, and are not intended to have biological activity. And therefore I don't think it's unreasonable to go looking for compounds which give very low response in the biochemical test, even at repeated doses. However, when we turn to pesticides, I'm not sure that the same rigid rule can be applied. After all, to be a pesticide, an organophosphorus ester has got, first of all, to have an ability to inhibit esterases. It's tailored to inhibit acetylcholinesterase of the pest in particular. It is always likely that it will have some activity against other esterases. I think it is asking the impossible of the chemists and of enzymology and of proteins, to imagine that you could ever get a pesticide which would be highly potent against pest acetylcholinesterase and inhibit neither mammalian acetylcholinesterase nor any other esterase to any degree whatsoever. That's asking for the moon. What is done, is that the compounds are tailored to have comparative toxic responses, high anti-pest activity and lower mammalian toxicity. And we can do something, in similar style, about segregating anti-cholinesterase activity from anti-neurotoxic esterase activity.

Table 2 is a summary of tests I have done measuring both the clinical and enzymic response in hens after administration of a single dose of a variety of organophosphorus esters (usually in conjunction with prophylactic and therapeutic treatment for anticholinesterase effects). Clinical ataxis was never seen at 10-20 days after dosing without a concomitant high inhibition of neurotoxic esterase at 1-2 days. The general impression emerges that phosphonates (and their thio-precursors) have a higher tendency to be neurotoxic than are phosphates. This is borne out by observations of inhibitory power of analogous structures in vitro where the phosphonates are more active against neurotoxic esterase by a factor which considerably exceeds the factor of increased lability or general reactivity: this trend is not true for anticholinesterase activity of phosphates and phosphonates.

When we turn to consider the intrinsic activity in vitro of a compound against neurotoxic esterase then factors such as metabolic disposal and anticholinesterase activity do not interfere with measurements. However one metabolic factor must be considered and this is activation. Table 3 shows that the two thiophosphonates illustrated, although neurotoxic in the hen, are not active against the enzyme in vitro whereas the oxon analogue is highly active both in vitro and in vivo. It is well known that anticholinesterase activity of thiophosphates is likewise negligible until the molecule has been activated by conversion to the

CLASS	Inhibition 70 - 100%, Ataxia Seen	Inhibition 0 - 70%, No Ataxia
(A) Acutely Toxic		
Phosphates, Phosphorothioates	9	20
Phosphonates, Phosphonothioates	14	5
Others	5	3
(B) Low Toxicity		
Tri-aryl phosphates	9	22*

* Some cause high inhibition and ataxia on repeated dosing

Table 2 General summary of the results of biochemical and clinical monitoring of organophosphorus esters for delayed neurotoxicity. Inhibition of neurotoxic esterase was measured 18-40 hours after dosing and clinical assessment of surviving hens was made daily until 21 days after dosing.

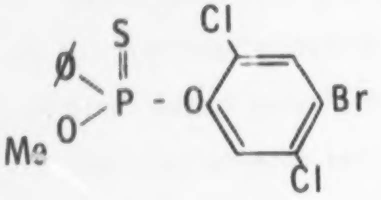
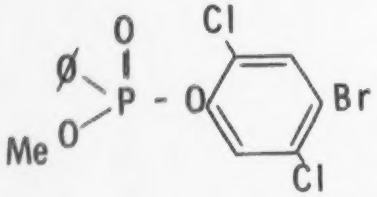
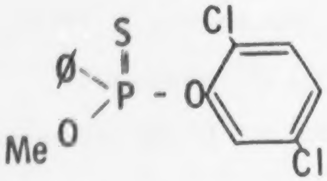
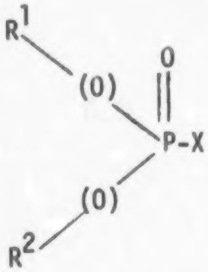
	Neurotoxic Dose (mg/kg)	In Vitro N.T.E. I_{50} (µM)
	400 - 500	N.S. (> 100)
	40 - 60	0.4
	60 (max)	N.S.

Table 3 Comparison of the effectiveness of typical neurotoxic thiophosphonates and a related oxon *in vivo* and *in vitro*. NTE = neurotoxic esterase; NS = not significant at 100 µM.

oxon. Thus it is not easily possible to start assessing neurotoxic potential of an organophosphorus ester by an exclusively *in vitro* test unless the major probable antiesterase metabolites are available. In most cases the identity of these is easily predicted, though DEF (S,S,S-tributyl phosphorothiolate) is one example which comes to my mind where I'm not sure how much is known of active metabolites. It is possible that a combined liver microsomal metabolising system could be included in an *in vitro* test system provided that interfering liver esterases were effectively inhibited.

Let us now dissect the influence of the substituents in a typical organophosphorus ester with the general structure



If the detailed data in references (1) and (2) are analysed we can make the further general observations:-

1) Phosphonates are more active than analogous phosphates while phosphinates are yet more active but are, of course, protective rather than neurotoxic as pointed out in the Mechanism talk.

2) Table 4 shows the marked increase in anti-neurotoxic esterase activity *in vitro* and *in vivo* as the bulk and hydrophobic character of R^1 and R^2 is increased up to the bis-*n*-pentyl homologue.

2,2'-Dichlorovinyl Phosphates

	In Vitro I_{50}(μM)	sc Dose (mg/Kg)	% NTE Activity remaining
Me	70	30	64
Et	2	18*	25
n. Pr	0.05	2*	5
n. Pent	0.003	2*	9
n. Hexyl	0.015		
n. Octyl	0.07		
n. Decyl	0.5 - 1		

* Neurotoxic dose

Table 4 The relationship of alkyl chain-length to neurotoxic potential assessed in vitro and in vivo. The I_{50}s refers to neurotoxic esterase not to acetylcholinesterase.

The same trend is seen in Table 5. The non-neurotoxicity of dimethyl phosphates is very marked. Joining the R^1 and R^2 substituents into a cyclic structure appeared to reduce neurotoxic potential in one compound I tested.

3) A comparison of Tables 4 and 5 shows the large effect of the leaving group (X). Thus the activities *in vitro* of the appropriate *n*-propyl compounds differ by a factor of 2000 and this is reflected (with a smaller factor) in their behaviour *in vivo*.

4) The most neurotoxic esters tend to have rather small and flexible leaving groups (X) such as fluoride or dicholorovinyl. Leaving groups which tend to lower neurotoxic potential include 4-nitrophenyl, oxime groups, a variety of heterocyclic groupings and groups with considerable steric hindrance around the ester bond.

5) Linkage of the leaving group to phosphorus via a thioloester bond rather than by oxygen drastically reduces the potency of inhibitors in the substrate and carbamate series and seems likely to do the same in phosphates.

6) Insertion of hydrophilic substituents into an aromatic ring also reduces inhibitory power against neurotoxic esterase.

7) Use of R or X groups which are non-planar (cyclohexyl), heterocyclic (pyridinyl) or very bulky (naphthyl, etc.) tends to produce compounds which do not act as good substrates or inhibitors of neurotoxic esterase.

Dialkyl 4-Nitrophenyl Phosphates

	In Vitro I_{50}(μM)	Dose (mg/Kg)	% NTE Activity remaining
Me	> 100	12 sc	100
Et	> 100	15 sc	88
Iso. Pr.	> 100	15 sc	99
_n_. Pr.	100	25 sc	62
_n_. Bu.	6	30 sc	52
2-Cl.Et	10	100 oral*	8

* Neurotoxic dose

Table 5 The relationship of alkyl chain-length to neurotoxic potential assessed _in vitro_ and _in vivo_. The I_{50}s refers to neurotoxic esterase not to acetylcholinesterase.

Many more potential pesticides have been synthesized in commercial research laboratories than ever reach the market. If these were re-evaluated by in vitro testing against neurotoxic esterase much more detailed structure/activity relationships could be worked out than those which I have sketched. Comprehension of the target should enable a rapid advance in synthesis of compounds concerning whose neurotoxic potential we can have quantitative data and, hopefully, a firm assurance of non-neurotoxicity. This is a great step beyond the assertion that "it didn't paralyse the hens".

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THE PATHOLOGY OF DELAYED NEUROTOXICITY DUE TO ORGANO-PHOSPHATES

W. A. Bradley, B.V.Sc.

Delayed neurotoxicity caused by organo-phosphorus compounds has been seen in many thousands of people. The most common group of compounds producing this are the tri-cresyl group of phosphates containing ortho-cresyl groups which have produced on occasions, epidemic-like outbreaks. Much of the work identifying the various members of this group that are neurotoxic was done by Bondy et al (1960), with the original work on tri-orthocresyl phosphate (ToCP) done by Smith and his co-workers. Smith and Elvove (1930) showed that Ginger Paralysis was caused by ToCP and that similar symptoms, including histopathological lesions, could be re-produced in the chicken (Smith, Engel and Stohlman 1932).

Other organo-phosphorus esters, particularly some of the fluorine-containing esters, can also produce neurotoxicity. Many of these are powerful cholinesterase inhibitors and as such, in some cases of poisoning, the victims do not often survive the initial crisis. However, reports have appeared showing that on occasions where the person has survived the initial exposure, delayed neurotoxicity has been seen. In 1953 Bidstrup, Bonnell and Beckett reported on two cases caused by Mipafox (bis-isopropyl aminofluorphosphine oxide) and Namba et al (1971) reported on a case where the person involved had worked on the synthesis of new organo-phosphorus compounds for 10 years. Generally, cases of delayed neurotoxicity are rare after poisoning with commercial pesticides, although haloxon (di-2-chlorethyl-3-chloro-4-methylcoumarin-7-yl phosphate) has produced neurotoxicity in the pig after an apparently normal anthelmintic medication (Christian, 1975).

Ever since Smith, Engel and Stohlman (1932) reproduced neurotoxic lesions in the hen which resembled those seen in the human with roughly a similar dose range, the hen has been the species of choice. Other advantages are that there appears to be no strain differences, the hen is easily obtainable, easy to keep in fairly basic surrounds and responds to a neurotoxic dose within a fairly short time (under 3 weeks usually). While results generally are easily reproducible and predictable, we have had two cases in our laboratories, using tri-aryl phosphates, where we have had a more severe reaction than expected. One of these had multiple fibrosarcoma nodules involving abdominal organs and tissues including the liver and the other had multiple caseous nodules (cause not diagnosed) throughout the liver. However, two cases are not sufficient to draw any definite conclusions as it is possible to account for these as being at the extreme of individual variation.

Apart from the hen, several other animal species have been shown to be affected and these have included cattle, goats, pigs, cats, some primates, pheasants and ducks. Other species such as the rat, rabbit, partridge and quail, appear to be resistant (Johnson 1975a).

Barnes and Denz (1953) showed that there was an age susceptibility in that young birds tended to be more resistant, although Johnson and Barnes (1970) were able to overcome this resistance with repeated doses. It is, therefore, advisable to use older birds. The United Kingdom

Ministry of Agriculture, Fisheries and Food recommend hens older than nine months for testing pesticides for neurotoxicity. However, when looking for very low levels of neurotoxicity it may be advisable to use birds in the two to three year-old age group. In fact I have been able to show very low levels of toxicity using older birds instead of younger ones and the results have correlated very well with those obtained by Johnson (1975b).

Following poisoning in hens with a neurotoxic organo-phosphorus compound there is initially, depression of cholinesterase and related enzymes which will produce effects ranging from no apparent clinical abnormality to sudden and rapid death. Assuming the hen survives the initial crisis, signs of neurotoxicity are seen generally between 8-10 days (range 7-20 days) after the neurotoxic dose. There does appear to be a critical dose level below which, signs do not appear but these lower levels do seem to be cumulative if repeated, although below an even lower level no toxicity can be produced (Smith, Engel and Stohlman 1932). Clinically the neurotoxic symptoms in hens appear to be confined mainly to the legs. Initially the birds appear to sit longer and when disturbed, a very slight unsteadiness in their gait is occasionally seen. When a dose above the minimal toxic dose is given the ataxia slowly develops. The tail tends to become depressed slightly so as to maintain balance, then walking becomes difficult and they stand with their hocks resting on the ground. Eventually they are unable to use their legs for walking though these are not completely paralysed and can still be vigorously moved. This phase takes up to two weeks to develop after the initial symptoms have been seen. If left the hens may die, usually as the result of starvation. Indeed, if body weight is monitored fairly carefully, slight weight loss is noticed from about the tenth day, particularly in birds that are kept in groups. There are two reasons for this, one being the increasing difficulty in getting to food and the other is the change in 'peck order' because of their disability. More often, providing they can obtain food and water, there will be a variable period when there is no apparent recovery or further deterioration before some recovery is seen. The less severe cases may recover completely, the others will show varying degrees of spasticity and ataxia.

Basically the lesion is a 'dying back' process or Wallerian degeneration in the axons (Figure 1) although earlier work tended to consider it a de-myelinating disease (Smith and Lillie 1931). Lesions are not detected histologically until after the delay period which is about the same time that clinical symptoms are noted. No changes are found before this time even when using electron-microscopy (Bischoff 1970). These are seen in the long sensory and motor pathways of the spinal cord and in the larger fibres of the peripheral nerves and restricted to the distal end of the axons. In the peripheral nerves, degenerative lesions are most often seen in the more distal muscles involving the intramuscular nerve bundles and the sensory nerves from the muscle spindle. In the hen lesions are very rarely seen much above the division of the sciatic into the tibial and peroneal nerves. The pathology of these lesions has been reviewed recently (Cavanagh 1973, Le Quesne 1975) and the distribution of the lesions, particularly in the C.N.S. was covered

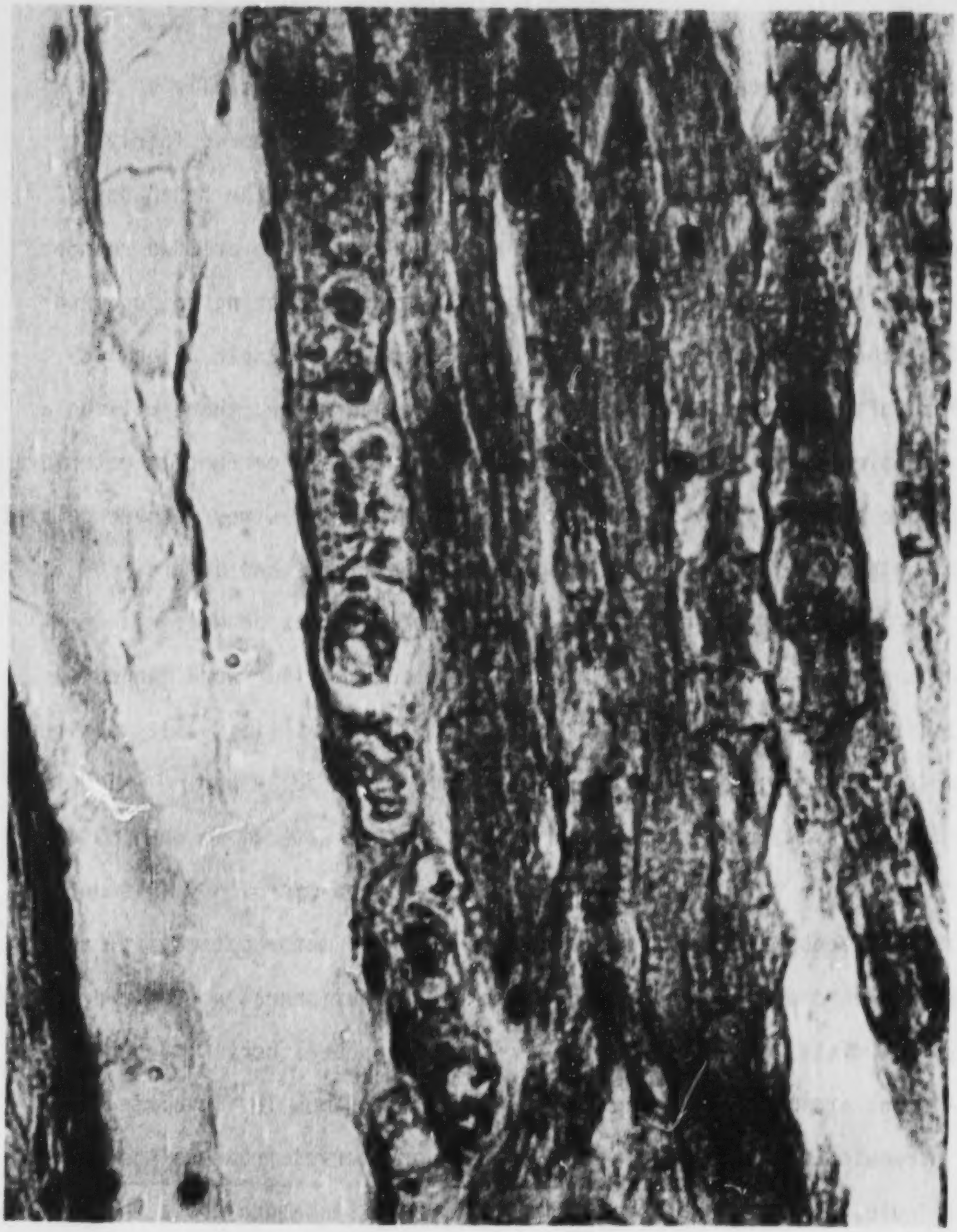

FIGURE 1. Wallerian Degeneration in a Nerve Fibre in the Tibial Nerve.
L. S. . H. and E. Stain Magnification 1200

earlier by Barnes and Denz (1952). Various methods for investigating and identifying neurotoxic lesions have been described by Glees (1967). Other degenerative lesions have been noted in other parts of the peripheral nervous system (Vij and Kanagasuntheram 1972; Krishnamusti, Kanagasumtheram and Vij 1972).

The neuroanatomy of the fowl has been described by Jungherr (1969). The dorsal funiculus carries mainly ascending fibres, the ventral funiculus, particularly near the ventral median fissure, has both descending and ascending fibres and the lateral funiculi tend to have ascending fibres in their lateral aspect and descending ones in their medial aspect. Histologically neurotoxic lesions are seen most easily in the long sensory and motor tracts at the distal end of the axon. Hence lesions in the ascending tracts are seen in the cervical region and in the descending tracts in the dorsal and lumbo-sacral region of the spinal column as well as in the more distal portion of the peripheral nerves (Figures 1, 2, and 3). Practically lesions have been seen at all levels of the spinal cord but with a preponderance in the upper and mid-cervical region. Lesions in the lumbo-sacral region tend to be fairly sparse. Janyck and Glees (1966) found chromatolysis of the spinal neurone in the lumbo-sacral region but generally this is not often seen.

Very rarely similar changes are seen in odd axons in normal hens. These can be distinguished from the neurotoxic lesions in that they are usually a single degenerating axon, often at a difference stage of degeneration, whereas neurotoxic lesions are usually multiple, bilateral in distribution and all of a similar 'age'. These odd single lesions are more likely to be in older birds where the occasional neurone dies off.

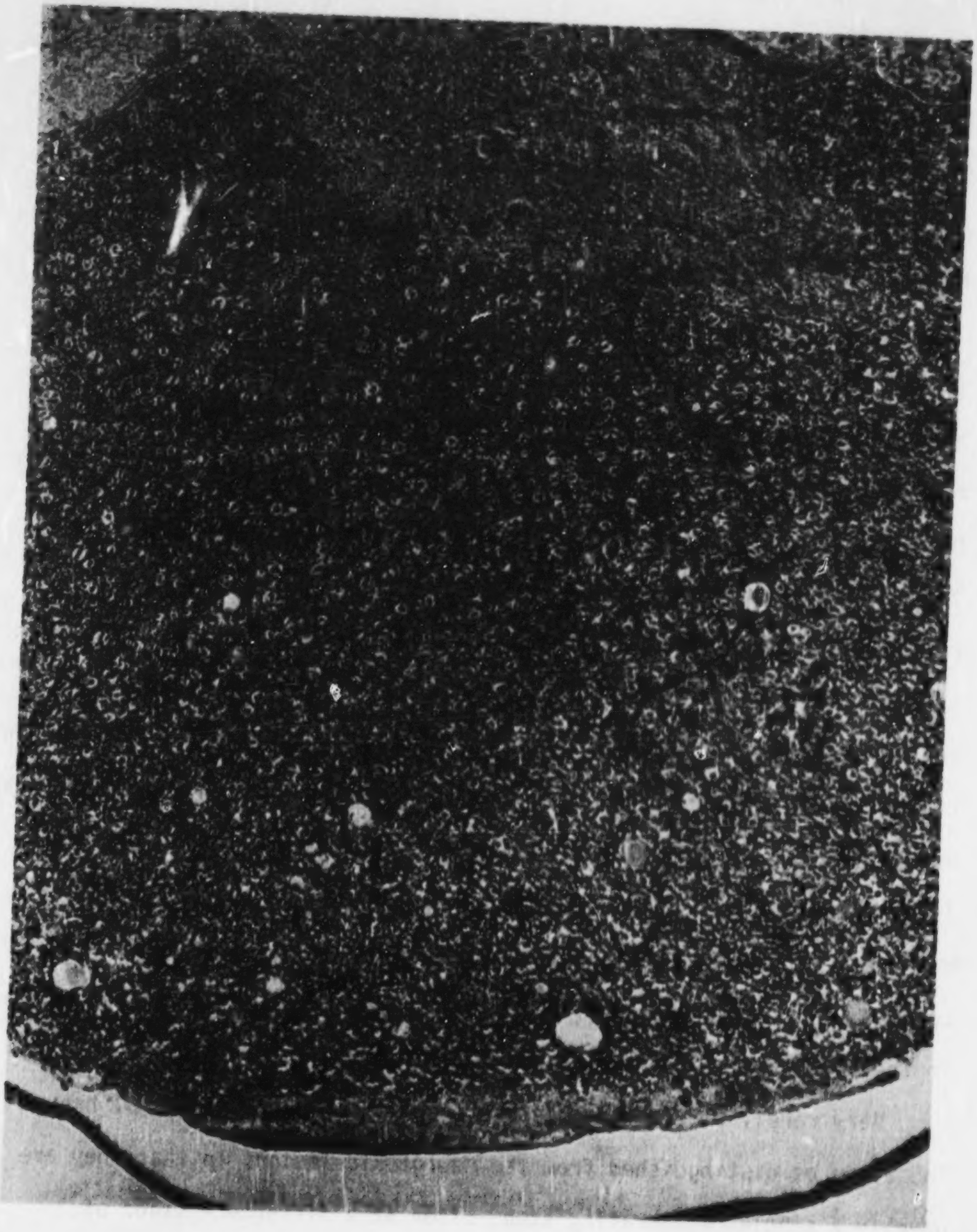

FIGURE 2. Degenerative Lesions in the Ascending Tracts of the Lateral Funiculus at an Approximate Level of C6. T.S.. H. and E. Stain Magnification 120

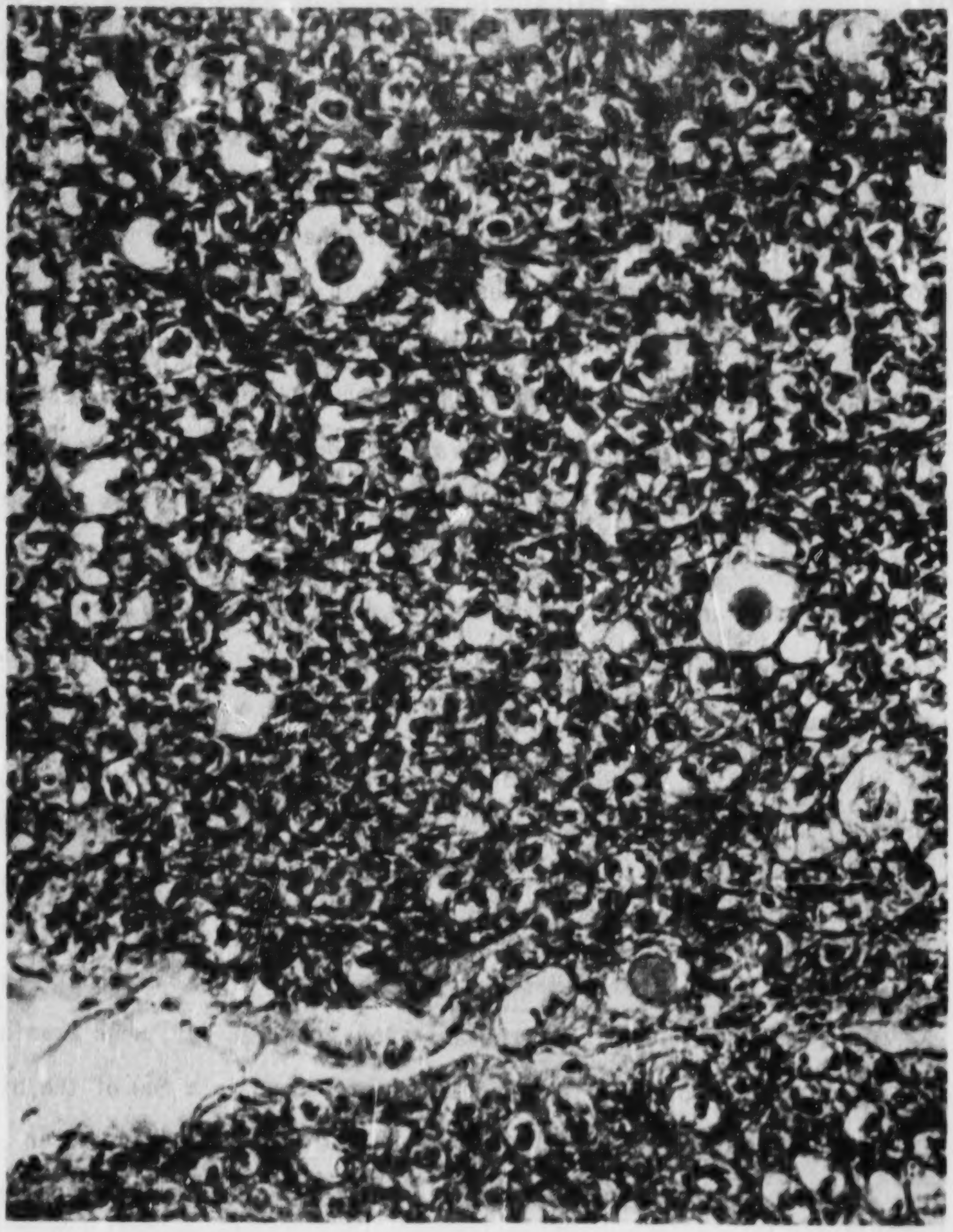

FIGURE 3. Degenerative Lesions in the Descending Tracts of the Ventral Funiculus at an Approximate Level of L. S. 1. T. S., H. and E. Stain Magnification 425

While specialised histological techniques have been described for examining nervous tissue, these are often time consuming and difficult. Hence they are not really suitable for regular examination of tissues from a number of birds, such as occurs in a routine toxicological study. Fortunately haematoxylin and eosin is one of the better stains for showing axon degeneration. The lesion seen in the spinal cord is swelling of the axon which often breaks up to form globules as seen in longitudinal sections (Figure 4). Those seen in transverse sections vary in the size and the amount of degenerating axon that they contain, this being dependent upon where the axon was sectioned. The lesions tend to be solid eosinophilic masses, occasionally slightly basophilic and sometimes foamy and faintly eosinophilic. In the peripheral nerve the lesions are not so obvious, there is not so much swelling and where the axon has degenerated it usually forms several small spherical eosinophilic masses which do not differentiate well from the surrounding tissue (Figure 1). When ataxia is present, histological lesions should always be found at some level in the cord and possibly in the peripheral nerves, unless some other cause for the ataxia is demonstrated. However, with compounds that are of low neurotoxic potential and others dosed at minimal effective doses, clinical symptoms are often not seen but, when examined histologically, typical bilateral lesions are found in a few of the birds.

In our laboratories, all birds are killed twenty-one days after the final dose as it is considered that any ataxia developing after this time is not usually caused by the compound being tested (Johnson 1975a). In looking for lesions it is important that good sampling from the spinal

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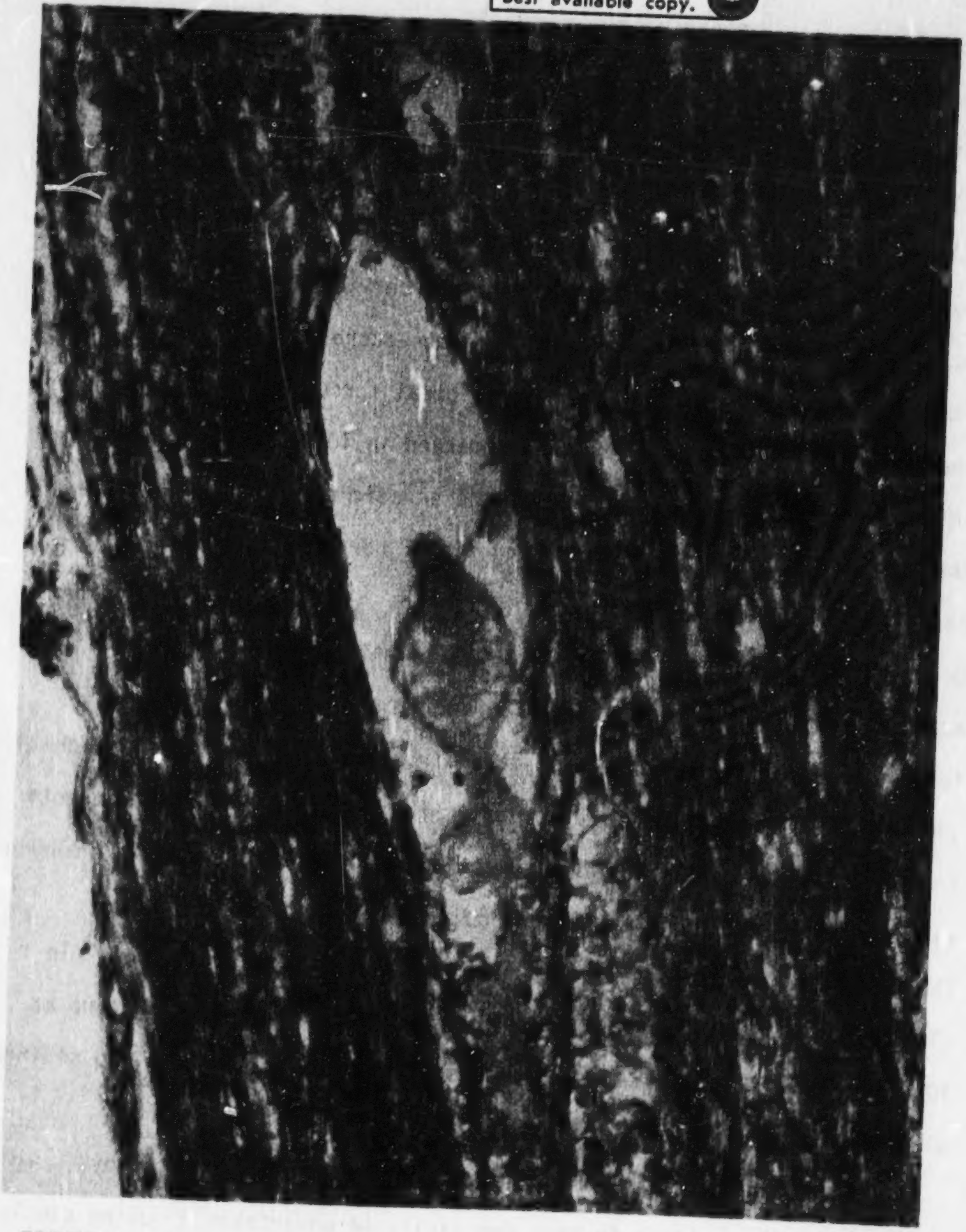

FIGURE 4. Degenerative Lesion in the Ascending Tracts of the Lateral Funiculus at an Approximate Level of C7-8. L.S.. H. and E. Stain Magnification 425

cord is carried out. To do this we anaesthetise the hen with pentobarbitone sodium given intravenously, and kill by exsanguination after cutting the posterior vena cava. We then perfuse with warm 10% formal saline (approximately 40°C) until the musculature stiffens. The spinal column is dissected out, with as much muscle as possible trimmed away, and divided into five segments as follows: cervical region into three equal parts, the dorsal region and the lumbo-sacral region (Table 1). Small nicks are made into the vertebral canal through the inter-vertebral spaces and the whole column is then immersed in 10% formal saline for at least seven days. The distal end of the sciatic nerve, together with the proximal part of the peroneal and tibial nerves are dissected from each leg, laid on card and fixed in formalin. After fixing, the spinal cord is carefully dissected out from the spinal column and each section, apart from the lumbo-sacral region and peripheral nerve, is sampled as follows: a transverse section is taken from close to each end of each segment and a longitudinal section cut through the lateral spinal tracts from the centre of each segment. The lumbo-sacral region has two transverse sections taken, the most distal one being through the glycogen body. The peripheral nerve is sectioned longitudinally as distal as possible (Table 2). Three sections from each level are then cut by microtome at 10 μm with 15-20 μm between such each section. These sections are stained with haematoxylin and eosin (Ziegler 1944).

The routine examination for delayed neurotoxicity produced by organo-phosphorus esters in hens can easily be undertaken by using simple clinical observations and fairly simple routine histological techniques. By using these methods with tri-aryl compounds containing ortho-cresyl groups,

Table 1. Vertebral Column of the Fowl

Region	Number of Vertebrae
Cervical	13 - 14
Dorsal (Thoracic)	7 No. 2-5 anchylosed; 7 anchylosed to lumbo-sacral
Lumbo-sacral	14 All anchylosed
Coccygeal	6 - 7 No. 1 anchylosed to lumbo-sacral

Table 2. Sampling Levels for Section

Region	Approximate Level Sections are Taken
Transverse Sections	
Cervical	1, 4, 6, 9, 10, 13.
Dorsal	2, 6
Lumbo-sacral	1, 5
Longitudinal Sections	
Cervical	2-3, 7-8, 11-12.
Dorsal	3-4
Peripheral Nerve	Proximal end of the tibial and peroneal nerves.

we have been able to pick up esters showing the whole range of neurotoxicity and where alternative biochemical methods have been used these have confirmed our findings (Johnson 1975b). However, with many pesticides organo-phosphates difficulties do arise in keeping the animal alive through the initial crisis caused by the depression of cholinesterase. Oximes and atropine are usually given to try to prevent death and the more severe symptoms. Thus the neurotoxicity of some of these compounds with very severe anticholinesterase activity may not be important unless it can be shown that repeated exposure to a small non-fatal dose can produce lesions of neurotoxicity.

Summary

A brief description of the neuropathology of delayed neurotoxicity due to organo-phosphate esters in the hen is given together with a method of sampling and examination that is suitable for routine examinations involving several birds.

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PERSISTENT EFFECTS OF ORGANOPHOSPHATE EXPOSURE AS EVIDENCED BY ELECTROENCEPHALOGRAPHIC MEASUREMENTS

Frank H. Duffy, MD
James L. Burchfiel, PhD, and Van M. Sim, MD

Introduction

Organo-phosphate (OP) compounds are known to have potent effects upon the nervous system of animal and man. They were first introduced as chemical warfare agents during World War II (nerve gas) and are now among the more common pest control agents (insecticides). Their extreme toxicity appears to result from a persistent anticholinesterase action at both the peripheral neuro-muscular junction and the central cholinergic synapse. OP compounds are still used clinically in the treatment of glaucoma and myasthenia gravis where such a persistent action may be advantageous.

Central nervous system (CNS) symptoms of acute OP exposure range from irritability and difficulty concentrating at low doses to overt convulsions at higher doses. Acute behavioral effects are usually accompanied by marked changes in the electroencephalogram (EEG) consisting of desynchronization and increased fast activity (beta) at lower doses and spike-wave discharges at higher levels.

Low level sustained administration of OP compounds produces more subtle but definite effects. There may be difficulty sustaining attention associated with slowing of intellectual and motor processes. Feelings of being generally slowed down, confused, agitated, and tense are reported.[1] Grobe et al reported the additional symptoms of insomnia, excessive dreaming, emotional lability, increased libido, paraesthesias, visual hallucination, and tremor in subjects given daily injection of diisopropyl fluorophosphate (DFP).[2]

Grishon and Shaw reported that of sixteen patients with prolonged low level OP exposure, eight had memory difficulty, seven were severely depressed, five demonstrated schizophrenic reactions, and four had unstable emotional states. These lasted up to one year following cessation of the exposure.[3] Similar, but less severe, long-termed sequelae were reported by Tabershaw and Cooper.[4] Rowntree et al suggested that OP exposure may precipitate or worsen symptomatology in patients with preexisting psychiatric problems.[5]

Not as well studied are the long term effects of single or multiple but spaced exposures to OP compounds. Rapid treatment of an acute exposure with an anticholinergic compound (atropine) and a cholinesterase activator (2-PAM CL) is usually effective in treating all but the most severe exposures. It is generally believed that if the secondary effects of hypoxia can be avoided that symptoms and signs do not persist beyond the immediate exposure period. In most instances EEG abnormalities have been reported to disappear within two weeks of acute exposure or at termination of low level sustained exposure.[2,6,7] However, other studies indicate that the EEG effects of insecticide poisoning may persist for many weeks or months.[8,9] Metcalf and Holmes suggested that OP exposure may lead to chronic EEG changes. They examined EEGs from a group of industrial workers with a past history of insecticide poisoning, but with no recent exposures, and reported a high incidence of abnormal low-to-medium voltage slow-wave activity in the theta range (4-6 Hz).[10]

The present study was undertaken to search for and statistically verify long term effects of OP exposure upon the primate brain as measured by changes in EEG activity. The OP, sarin, was chosen for study as it was the OP agent with the highest incidence of exposure in the Metcalf and Holmes study[10] (personal communication). Sarin (GB; isopropylmethylphosphonofluoridate), is not used commercially. It is ordinarily classified as a "nerve gas," however, it shares a similar chemical structure and pharmacological action with more common OP insecticides such as malathion and parathion. Sarin differs from most commercail OP compounds in two major aspects. First, it is fluorinated, and, second, it is considerably more potent.

A two part study was undertaken. First, rhesus monkeys were exposed to sarin. EEGs taken before and one year after exposure were compared for each animal. The number of exposed animals showing significant EEG changes at one year was compared to the number of unexposed (control) monkeys showing such changes (if any).[11] Second, the EEGs of a population of industrial workers with histories of exposure to sarin were compared to a control population, (report in preparation).

Methods

A) Monkey Study

Twenty-two rhesus monkeys of both sexes, weighing 2.5-4.0 Kg were used in this study. Dural EEG electrodes were placed (under general anaesthesia and asceptic conditions) over the frontal, central, occipital and temporal cortices, bilaterally. Insulated leads from the electrodes were soldered to two 9-pin Winchester-type miniature connectors which were cemented to the skull with dental acrylic. Additional acrylic was used to form a durable, protective cap over the skull covering all leads and electrodes.

Sarin for injection was prepared by dilution of an aliquot of purified drug (stored at-55°F) with normal saline, Biological effectiveness of this solution was determined routinely in rabbits and was never used more than 2 hr after dilution. Chemical analysis of random samples indicated that sarin was always 90-95°/₀ pure.

Sarin was administered according to one of two schedules. In one, animals received a single "large dose" of the drug: 5 ug/kg of sarin i.v. In the other, animals received multiple "small doses": a series of 10 injections of 1 μg/kg of sarin, im, given at intervals 1 week apart. Hereafter these will be referred to respectively as the "large-dose" and "small-dose" schedules. These dose levels were arrived at through pilot studies in which the attempt was made to find dose levels which would approximate, respectively, (1) a serious but sublethal exposure showing all the signs of poisoning and (2) a series of subclinical but near-threshold exposures showing few, if any, signs of overt poisoning.

At the large-dose level all animals had generalized seizures. To prevent the possibility of secondary anoxic brain damage, animals were paralyzed with gallamine triethiodide, and artificially respired. Sarin was given by slow iv injection over 3 min. Animals were allowed to recover from paralysis and breathe spontaneously when their EEG revealed a cessation of seizure activity. The average total length of paralysis for experimental animals was about 2.5 hr and never more than 4 hr. A group of control animals was similarly paralyzed and received an injection of an equal volume of drug diluent. The length of paralysis for controls was matched as nearly as possible to that of the experimentals. Paralysis was not necessary for the animals receiving the multiple small doses.

Animals were randomly assigned to one of two drug-treatment groups: (1) single, large-dose sarin; (2) multiple, small-dose sarin. There were three animals in each treatment group. These groups were matched with groups of control animals. Controls were subjected to the same injection schedules.

Each animal in the study was subjected to a series of EEG recording sessions. Prior to drug administration, three separate control sessions were performed on different days. For the single, large-dose animals, a recording was taken 24 hr after administration. For the multiple, small-dose animals, a recording was taken 24 hr after administration of the last injection in the series. Subsequently, the EEGs of all animals were periodically monitored to check the integrity of the recording system and CNS activity. One year after exposure an additional three recordings were performed for all animals on three separate days. During the 1-yr interval the animals were maintained in separate cages in a temperature-controlled environment; they received no other drugs.

During a recording session the animal sat in a primate-restraining chair, with a mechanical head restraint, inside a closed, electrically shielded chamber. EEGs were recorded on seven-channel FM magnetic tape (AMPLEX FR-1300) via calibrated outputs of a Grass Model 78 polygraph with a bandpass of 0.580 Hz. An individual recording session consisted of the following runs: (1) Awake, alert in light, an epoch of at least 2-min duration in which the animal sat in a lighted chamber; alertness was maintained by intermittent auditory distraction; (2) Awake, alert in darkness, same as (1), but in darkness; (3) drowsy, an epoch of 15-30 min duration in which the animal sat in the darkened chamber and silence was maintained.

For each run, recordings were obtained from the cortical leads in the following bipolar pairings: frontal-central, central-occipital, and occipital-temporal.

Spectral analysis of the EEG data was accomplished on a PDP-12 computer (Digital Equipment Corp.) using the comprehensive SIGSYS-12 software system (Agrippa Data System-Boston). Signals were played back from FM tape through a Khrone-Hite filter set to bandpass 0.5-50 Hz (24 db per octave) and analog-to-digital converted by the computer at a rate of 256 Hz. Fast-Fourier transforms (FFTs) were performed, without smoothing, on 4-sec epochs of EEG (Computed on 1024 points over the frequency range 0-128 Hz with a resolution of 1/4 Hz). After FFT computation, the square root was taken of each frequency component to yield a voltage spectrum. Epochs containing excessive eye blinks, movement artifact, electrode artifact, or muscle activity were not analyzed. Consecutive epochs were averaged to yield a spectrum representing 1 min of EEG; i.e., the spectra of 15 4-second epochs were averaged. Two 1-min spectra were computed for each state in a recording session (i.e., awake in light, awake in darkness, and drowsy). The 2-min epoch of EEG most representative of drowsiness was selected by visual inpection of the polygraph tracing obtained in parallel with the analog tape recording. The selection of the drowsy epoch was made by an experienced electroencephalographer (EEGer), based on the monkey EEG criteria of Reite et al.[12] The EEGer was unaware of a monkey's exposure history at the time of selection.

For each spectrum, a calculation was made of the percentage of total energy present in each of the classical EEG frequency bands defined as follows: delta=0.5-3.75 Hz, theta=3.75-7.75 Hz, alpha=7.75-13.0 Hz, beta 1=13-22 Hz, and beta 2=22-50 Hz. The band from 50 to 128 Hz was not analyzed.

For the final analysis, total spectra and frequency bands were averaged respectively for the three pre-drug sessions, the 24-hr session, and the three 1-yr sessions. This was done for each lead and each state. Thus, a final datum consisted of an average spectrum and frequency bands representing EEG activity from one electrode derivation, for one state, and for one set of sessions. Since two spectra were derived for each state and lead in a recording session, the values of N for the final averages were 6 for pre-drug, 2 for 24 hr, and 6 for 1 yr. In computing the final averages, standard deviation was also calculated.

Statistical analysis was performed in a two-step procedure. First a longitudinal comparison was made for each animal using a two-tailed Student's t test. The average spectrum and frequency bands derived from the control sessions (for a given lead and state) were compared first with that derived from the session at 24 hr post-drug and next with that from the sessions at 1 yr. For the average spectrum, the t test was applied at each point. As might be expected, this produced a large number of seemingly random "significant" differences. (Theoretically, one would expect an average of 5% of the t tests to yield t values with a probability ≤ 0.05 merely by chance.) Such differences at individual points throughout the spectrum proved very difficult to interpret. We felt that any criteria we might establish to determine when statistically significant points constituted a physiologically significant difference might suffer from subjective bias. For this reason we chose to base our results upon analysis of integrated frequency bands. Therefore, at the conclusion of the first step of statistical analysis, we had determined for each animal which frequency ranges were different by t test at 24 hr post-drug and at 1 yr post-drug as compared to the pre-drug control.

In the longitudinal comparison, each animal served as his own control; data from animals within the same experimental group were not pooled for comparison. The next question, therefore, was which differences, if any, revealed by t test were consistently and exclusively seen among the drug-treated animals as opposed to control animals. This question was answered by the second step of the statistical analysis, Fisher's test.[13] For this analysis the question was asked, how many drug-treated animals showed a significant change (by t test) in a particular frequency band and how many did not? The same question was asked of the control animals. From these answers a 2 x 2 contingency table was constructed and the exact probability of obtaining the resultant distribution in the table, or one more extreme, by chance alone was calculated. If this probability were ≤ 0.05, then it was concluded that a significant change in frequency distribution of the EEG had occurred in a drug-treated group as compared to the controls. Statistical analysis among the various groups of control animals showed no consistent differences; therefore, for Fisher's test all control animals were considered as a single group.

B) Human Study

Three separate but related investigations were undertaken: (1) spectral analysis of tape recorded EEG, (2) visual inspection of routine clinical EEG, and (3) visual inspection of an all night sleep EEG.

Seventy-seven industrial workers with histories of accidental exposure to sarin were studied. Thirty-seven of these had 3 or more exposures within the 6 years preceeding the study but no subject was exposed during the year just preceeding the study. The exposure criteria were defined as a history of exposure, with resultant clinical signs and symptoms, and a demonstrable reduction in red blood cell cholinesterase to a level at least 25% of the individual's pre-exposure baseline.

Thirty-eight workers from the same geographical area with no history of OP exposure (chosen so that their age distribution, sex, race, and socioeconomic background matched that of the exposed subjects)-were used as controls. Until it became necessary to group the subjects for statistical purposes, no one involved in data analysis knew the personal or exposure history of the individual subjects.

Subjects were studied in a standard EEG laboratory. EEG activity was recorded on an Ampex FR 1300 tapedeck via a Grass model 78 polygraph with a bandpass of 0.1-35 Hz. A simultaneous strip chart was made to monitor for artifact and to assess level of consciousness. Subjects were studied in the following states:

1. Eyes open - EO (2 minutes) - awake with eyes open in a lighted environment and frequently alerted by an auditory stimulus.

2. Eyes closed - EC (2 minutes) - same as 1, but with instructions to close eyes.

3. Drowsy - D (15 minutes) - drowsiness and sleep. Room lights

were turned off and extraneous sounds were masked with white noise. One minute of this 15-minute period was chosen to represent drowsiness on the basis of the EEG strip chart. Drowsiness was defined as the state beginning after the break-up of alpha activity and ending with the early signs of vertex waves and spindling.

4. Hyperventilation - HV (5 minutes) - 5 minutes of deep breathing with eyes open and room lights on. The third minute was chosen to represent the HV state.

5. Recovery from hyperventilation - PHV (3 minutes) - the third minute following cessation of HV was used to represent the PHV state (eyes open and room lights on).

Off-line data analysis was performed on the EEG activity recorded from the following bipolar linkages (10-20 international electrode placement): O_z-C_z (occipital-central), F_3-C_3(frontal-central), and T_3-T_5 (temporal). Signals were bandpassed between 0.5 and 32 Hz (24 db per octave) at the time of computer analysis. Frequency spectra were formed for each subject, state, and linkage. Spectral analysis was performed in the same manner as for the monkey study with the exception that the analysis was limited to the 0-30 Hz range. The band from 30-128 Hz was not analyzed. For each spectrum the energy in ten 3-Hz segments was calculated from 0.5 to 30 Hz. These segments may be related to the standard EEG bands as follows:

Delta = 0.5-3 Hz, slow theta = 3-6 Hz, fast theta = 6-9 Hz, alpha =

9-12 Hz, slow beta = 12-15 Hz, beta = 15-30 Hz.

To facilitate analysis the energy within each frequency band was averaged across all subjects within a group for each state and linkage. Variance and standard deviations were also calculated. Comparisons were made between groups (for each state and linkage) on the basis of three statistical tests: Student's t-test, Mann-Whitney U-test, and chi-square analysis. For a test to be considered valid, it had to achieve the 0.05 two-tailed level of significance. The two non-parametric tests (U test and chi-square) were undertaken due to the relatively large variance encountered in the various groups which we felt might interfere with the parametric t-test. The u-test is sensitive to the relative rank of each member of a group but not its absolute value. Chi-square analysis was performed as follows: the combined mean of the two groups being compared was calculated; next, the number of members in each group above and below the mean was placed in a 2 x 2 contigency table and chi square was calculated. Due to the larger numbers in each cell (>5) we did not have to use the Fisher test to analyze the 2 x 2 table.

Routine clinical EEGs were performed using a Grass Model 78 EEG with a complete 24 electrode montage. Both monopolar and bipolar linkages were examined. In addition to the standard clinical "reading", the EEGer designated as "normal" or "abnormal" 17 separate parameters (see Table 6). The numbers of exposed workers or control workers within the normal-abnormal category for each parameter were placed in a 2 x 2 contengency table. Statistical comparison was made by Fisher's test as individual entries were often below 5 in number. At the time of evaluating the EEG, the EEGer was unaware of the subject's exposure history. Computer spectral analysis and visual readings were done at the Seizure Unit, Childrens Hospital Medical Center at Harvard Medical School, Boston.

All night sleep EEGs were performed during a subject's usual sleep period. Three bipolar linkages of EEG (Oz-Cz, F3-C3, F4-C4), an electromyogram (EMG) of neck muscle tone, an electrooculogram (EOG) for eye motion potentials, an electrocardiogram (EKG), and a bed movement transducer were recorded on the paper strip chart of a Grass Model 78 polygraph. These charts were then sent to the Sleep Laboratory of the Univ. of Maryland (Dr. Althea Wagman) in Baltimore for scoring. Absolute and percent times in rapid eye movement (REM), stage 1, stage 2, stage 3, and stage 4 sleep were calculated for each subject. Comparisons between groups were made by t-test and u test.

For all analyses, control subjects were compared to all the exposed subjects (CxE analysis) and again to the SVB-group of subjects with multiple (3 or more) exposures (CxM analysis). All data analysis was performed at the Seizure Unit with a PDP-12 computer using the parametric and non-parametric statistical packages of SIGSYS-12.

RESULTS

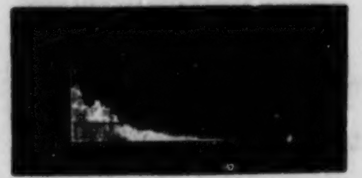
A) Monkey Study

Long term (one year) alterations in EEG frequency spectra were observed for both the single, large-dose and multiple, swall-dose exposed monkeys.

Large dose. The single large dose of sarin (5 µg/kg) produced a persistent increase in the relative amount of voltage in the beta frequency bands (13-22 and/or 22-50 Hz) of the occipital-temporal (O-T) EEG derivation. Figure 1 shows the data for a control monkey and Figure 2 for a large dose sarin animal.

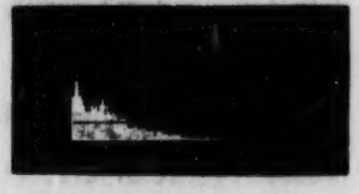
Figures 1 and 2 illustrate some general findings which are applicable to all the drug treatment groups. First of all, it can be seen in comparing the control records from these animals that a considerable degree of individual variability exists in the EEG frequency distributions. However, for a given animal, the spectra tended to be reasonably constant from day to day. The control animal shown in Fig. 1 is typical of the data from the 10 untreated animals. There are no significant differences among the spectra over the 1-yr period of the study. Because of the much greater degree of variability from animal to animal than from day to day for the same animal we adopted the longitudinal method of statistical analysis described in the methods section. Similar degrees of variability were seen among the drug-treated animals. However, the key factor among the large-dose sarin animals was that, regardless of what other alternations in the spectra might occur, there

CONTROL



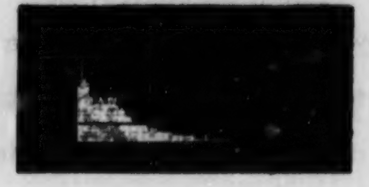
BAND	PERCENT ENERGY	S.E.
D	23.54	0.62
T	18.35	0.62
A	22.22	1.00
B1	18.47	0.77
B2	20.86	1.00

24 HOURS



BAND	PERCENT ENERGY	S.E.	t VALUE vs CONTROL
D	22.74	1.87	0.56
T	17.02	0.42	1.16
A	22.26	1.56	0.02
B1	20.68	0.53	1.55
B2	20.86	0.42	0.01

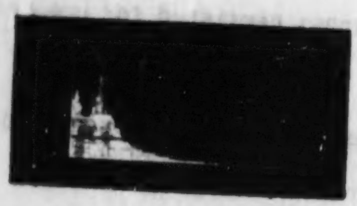
1 YEAR



BAND	PERCENT ENERGY	S.E.	t VALUE vs CONTROL
D	23.46	1.57	0.05
T	18.00	0.75	0.36
A	21.95	0.95	0.20
B1	20.14	0.97	1.34
B2	19.94	0.72	0.75

FIGURE 1. Example of average voltage spectra and frequency bands for one control animal. Occipital-temporal EEG derivation in the state of awake in darkness. For the spectra, the ordinate scale is in microvolts and the abscissa scale is in hertz. Frequency bands give percentages of total voltage (0-50 Hz) over the following ranges: D = delta (0.5-3.75 Hz); T = theta (3.75-7.75 Hz); A = alpha (7.75-13.0 Hz); B1 = beta 1 (13-22 Hz); B2 = beta 2 (22-50 Hz). The t value is the result of Student's t test between the indicated band and the corresponding band of the control spectrum., $P \leq 0.05^{*}$, $P \leq 0.01^{**}$

CONTROL

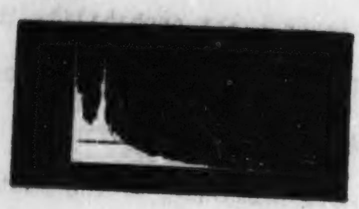


BAND	PERCENT ENERGY	S.E.
D	19.89	0.92
T	19.32	1.05
A	32.50	0.94
B1	18.72	0.78
B2	13.25	0.41

24 HOURS

BAND	PERCENT ENERGY	S.E.	t VALUE vs CONTROL
D	26.80	2.12	3.53**
T	19.05	0.65	0.14
A	19.39	0.33	7.64**
B1	19.65	1.44	0.59
B2	18.26	0.22	6.68**

1 YEAR



BAND	PERCENT ENERGY	S.E.	t VALUE vs CONTROL
D	21.05	0.29	0.99
T	17.16	0.73	1.51
A	30.36	0.93	1.55
B1	18.64	0.58	0.07
B2	16.29	1.23	2.77*

FIGURE 2. An example of voltage spectra and frequency bands for a large-dose sarin animal. See legend, Figure 1. Note persistence of increased beta 2 at 1 year.

was a consistent increase of relative voltage in the beta frequency bands. For instance, in Fig. 2 at 24 hr post-drug there was a significant increase in beta 2 which was still present 1 yr later. But, in addition, there was a significant increase in Delta and a decrease in Alpha at 24 hr. Neither of these changes persisted for one year, however.

A summary of the results of t test analysis for increased relative beta activity in the O-T frequency spectrum is given in Table 1. Each entry in the table is the proportion of animals (control of treated) which showed a significant increase in beta activity by t test. For a given post-drug session and recording state, the proportion of treated animals showing an increase and the proportion of controls showing an increase form a 2 x 2 contingency table. Beneath each pair of proportions, in parentheses, is given the Fisher exact probability that such a distribution could arise by chance alone (Where there is obviously no difference, the probability is omitted). It can be seen that the increase in relative beta voltage was most prominent in the state of awake in darkness. At 24 hr after administration, all three drug-treated animals showed a statistically significant increase in beta activity by t test, whereas only two of ten controls showed such an increase. The probability of this distribution arising by chance is 0.035. At 1 yr after sarin exposure two of three treated monkeys still showed a relative increase in beta activity; none of the controls showed any increase. This distribution has a chance probability of 0.0385.

TABLE 1

SINGLE LARGE-DOSE ADMINISTRATION OF SARIN, OCCIPITAL-TEMPORAL EEG[a]

	Recording state		
	Awake in light	Awake in darkness	Drowsy
24 hr			
Sarin	0/3	3/3	2/3
Control	0/10	2/10 (p=0.035)[b]	1/10 (p=0.105)[b]
1 yr			
Sarin	2/3	2/3	3/3
Control	2/10	0/10 (p=0.038)[b]	2/10 (p=0.035)[b]

---

[a] Sarin 5 μg/kg, iv. Proportion of animals showing a significant increase by t test in relative beta voltage. A percentage of total voltage of spectrum in the frequency bands 13-22 or 22-50 Hz of the EEG frequency spectrum at 24 hr and 1 yr post-drug.

[b] Exact probability of distribution of drug-treated and control animals occurring by chance (2x2 Fisher Test).

For the drowsy state there was a significant increase in beta activity for treated animals at 1 yr (all sarin animals showing an increase as opposed to two of ten controls, p=0.035). However, at 24 hr this change was not quite statistically valid (an increase in two of three treated animals and in one of ten controls, p=0.105).

In the state of awake in light, there was no significant increase of relative voltage in the beta bands. However, there would appear to be a trend toward increased beta at 1 yr after exposure (two of three treated animals showed a significant increase, while eight of ten controls did not, p=0.188).

Small dose. The animals receiving the multiple small doses of sarin (1 μg/kg once a week for 10 weeks) showed a persistent relative increase in O-T beta activity similar to that seen with the large-dose animals. The results of t test analysis for increased beta activity in all small-dose animals is given in Table 2. At 24 hr after the last dose of sarin, all the treated animals showed a significant increase in beta for both the states of awake in darkness and drowsy. These increases were still present at 1 yr after exposure.

In addition to the alternation in O-T EEG spectra, the small-dose animals also showed increases in relative beta activity in the frontal-central (F-C) EEG (Table 2). Such F-C alterations were not seen with the single large dose of sarin. The increase in F-C beta also differed from the increase in O-T beta in that it occurred only in the spectra at 1 yr post-drug; at 24 hr there was no significant increase (Table 2).

TABLE 2

MULTIPLE SMALL-DOSE ADMINISTRATION OF SARIN, OCCIPITAL-TEMPORAL (O-T) AND FRONTAL-CENTRAL (F-C) EEG[a].

	Recording state		
	Awake in light	Awake in darkness	drowsy
A. O-TEEG			
24 hr			
Sarin	0/3	3/3	3/3
Control	0/10	2/10	1/10
		(p=0.035)[b]	(p=0.014)[b]
1 yr			
Sarin	2/3	2/3	3/3
Control	2/10	0/10	2/10
		(p=0.0385)[b]	(p=0.035)[b]
B. F-CEEG			
24 hr			
Sarin	0/3	0/3	1/3
Control	1/8	2/8	1/8
1 yr			
Sarin	3/3	3/3	3/3
Control	1/8	1/8	1/8
	(p=0.024)[b]	(p=0.024)[b]	(p=0.024)[b]

---

[a] Sarin, 10 injections of 1 μg/kg, im, given at 1-week intervals. Also see footnote a, Table 1.

[b] See Footnote b, Table 1.

In contrast, the spectra at 24 hr after termination of the small-dose schedule showed a consistent trend for all treated animals toward increases in relative voltage in the delta and theta bands and decreases in higher frequencies, particularly alpha. With time this frequency distribution reversed such that beta was increased and delta and theta decreased. The increase in relative beta at 1 yr was seen in all three recording states (Table 2). In Table 3, all monkeys exposed to sarin are grouped and compared to the control animals for the O-T lead, beta range, one year after exposure. Note the high significance level for both states.

B) Human Study

(1) Spectral Analysis

Tables 4 and 5 show the results of the CxE and CxM analyses, respectively. Note in Table 4 that that there were significant decreases in delta in the Eyes Open (EO) state for the T3-T5 (temporal) and F3-C3 (frontal) linkages ($P<.02$). However, no other comparisons reached significance. This raises the important question of how many individual comparisons must be statistically significant to have physiological significance. For example, the T3-T5 linkage in Table 4 has 50 comparisons - 5 states and 10 bands. At the .02 level, .02 x 50 or 1 comparison might have shown significance by chance. For the entire C x E analysis of Table 4, .02 x 150 or 3 comparisons might show significance by chance. As only one comparison in T3T5, one in F3C3, and two in the entire C x E analysis were statistically significant we must conclude

TABLE 3

PERSISTENT EFFECTS OF SARIN EXPOSURE ON MONKEY EEG ONE YEAR AFTER EXPOSURE

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increased temporal lobe beta; (exact probability test)	exposed animals: control animals:	$\begin{array}{c\|c} \text{signif increase} & \text{no increase} \\ \hline \text{signif increase} & \text{no increase} \end{array}$			
	SMALL DOSE		LARGE DOSE		COMBINED	
EYES CLOSED DARKNESS	$\begin{array}{c\|c} 2 & 1 \\ \hline 0 & 10 \end{array}$	.039	$\begin{array}{c\|c} 2 & 1 \\ \hline 0 & 10 \end{array}$	.039	$\begin{array}{c\|c} 4 & 2 \\ \hline 0 & 10 \end{array}$	.008
DROWSY	$\begin{array}{c\|c} 3 & 0 \\ \hline 2 & 8 \end{array}$	.035	$\begin{array}{c\|c} 3 & 0 \\ \hline 2 & 8 \end{array}$	.035	$\begin{array}{c\|c} 6 & 0 \\ \hline 2 & 8 \end{array}$	.003

A combination of tables 1 and 2 for O-T EEG beta activity at one year Here, small and large dose animals are combined and compared to unexposed animals. Note the high level of significance for the confined comparison.

TABLE 4

CxE ANALYSIS OF SPECTRA

		.5-3	3-6	6-9	9-12	12-15	15-18	18-21	21-24	24-27	27-30	
T3T5	EO	T										
	EC											
	D											
	HV											
	p̄HV											
F3-C3	EO	TUC										
	EC											
	D											
	HV											
	p̄HV											
O2C2	EO											
	EC											
	D											
	HV											
	p̄HV											

Table 4

Results of the spectral analysis between the control subjects (c) and all exposed subjects (E). The ten columns represent the 10 integrated frequency bands from 0.5 to 30 kg in 3 Hz steps. The rows represent different recording locations and different clinical states. EO=eyes open alert, EC=eyes closed alert, D=Drowsy, HV=hyperventilation, pHV=after hyperventilation, T3T5=temperal, F3-C3=frontal-central, O2-C2=occipital-central.

Each table entry represents the significance level of the CxE comparison for that frequency band, lead, and state. The absence of an entry indicates failure to reach the .05, 2 tailed, level of significance. T=significant t-test, U=significant U-test, C=significant chi-square test. The arrow indicates the direction of the effect with reference to change from the C group mean.

In this table, eyes open, temporal, delta (0.5-3Hz) was significantly decreased by t-test in T3-T5 ($P \leq .02$) and by t-test, U-test and chi-square in F3-C3. No other comparisons were significant.

TABLE 5

CxM ANALYSIS OF EEG SPECTRA

		.5-3	3-6	6-9	9-12	12-15	15-18	18-21	21-24	24-27	27-30
T3T5	EO						T	T			
	EC						T	T			
	D						T	TU	T		TC
	HV										
	$\bar{p}$HV						T				
F3C3	EO										
	EC										
	D				C						
	HV								TCU	U	
	$\bar{p}$HV										
O2C2	EO									C	
	EC							TU	C	C	
	D						C	TC	CU		
	HV								TU	TCU	
	$\bar{p}$HV							TU	TUC	T	

Table 5

Results of the spectral analysis between the control subjects (C) and the maximally exposed subjects (M). Same convention as table 1. All significance levels are 0.05 2 tailed or better. Note the clustering in the beta range (13-30Hz) of T3T5 and OZ-CZ and the fact that all significant levels represent an increase of beta activity in the maximum exposure group.

that these may have arisen by chance alone and, therefore, have no definite physiological significance.

On the other hand, the C x M analysis of Table 5 shows a different picture. There are nine significant comparisons ($P \leq 0.05$) for T3T5, three for F3C3 and twelve for OZCZ. For each linkage we might expect 2.5 comparisons significant by chance at the .05 level. For the entire analysis we might expect 7.5 by chance. Clearly, we have exceeded the chance level for all linkages (especially T3T5 and OZCZ) and the entire C x M analysis (24>7.5). The physiological significance is increased by the clear tendency for the results to cluster in the beta range and to represent an increase of beta in every case.

Thus, the results of the spectral analysis show (a) no differences between the control and entire exposed populations (b) extremely significant differences between the control and maximum exposure populations, (c) a tendency of the differences to cluster in the beta range and represent an increase in beta within the exposed group.

(2) Visual Inspection of Clinical EEG

Table 6 shows the results of the C x E and C x M analyses for the parameters designated normal or abnormal by the EEGer. Note that there is a significant decrease in background EEG voltage at the .015 level and a decrease in alpha amplitude at the .04-.06 level for both analyses.

Thus the results of the visual suspection of EEG suggest a decrease of background voltage and alpha.

TABLE 6

HUMAN EEG READINGS-EXACT PROBABILITY TEST

	WAKING	DROWSY	SLEEP	HYPER-VENT'N	POST HYPER-VENTL'N	STROBE	ENTIRE RECORD	BKGND VOLTAGE	BKGND RHYTHMS
C	20/13	18/15	23/10	22/11	23/10	30/3	16/17	32/1	25/2
E	39/54	37/36	38/26	43/30	53/20	61/12	31/42	51/14	50/6
CxE	.1331	.1556	.1093	.1299	.1736	.1547	.1413	.0110**	.2890
M	13/14	14/13	14/9	15/12	19/8	22/5	10/17	17/6	16/4
CxM	.1306	.1987	.1781	.1439	.2221	.1722	.1409	.0144**	.1584

	PAROXYSMAL ACTIVITY	FOCAL ACTIVITY	ASYMMETRIES	DELTA	THETA	ALPHA	BETA	DOCTORS GUESS
C	24/9	32/1	30/3	30/3	21/11	27/6	24/9	19/14
E	45/28	70/3	72/1	58/15	42/31	48/25	56/17	36/37
CxE	.0971	.4132	.0802	.1331	.1286	.0458*	.1712	.1228
M	17/10	24/3	26/1	21/6	14/13	17/10	17/10	13/14
CxM	.1591	.1979	.3021	.1607	.1196	.0625	.1591	.1584

NORMAL/ABNORMAL

Table 6

Results of the Fishers exact probability test on the Human EEG data obtained by visual inspection of EEG. Numbers above represent the numbers of normally or abnormally classified subjects in each group. C = control group, E = entire exposed group, M = maximally exposed group, CxE = comparison between control and exposure group by Fisher's test C x M = same as C x E but for maximally exposed group. Exact probability values are shown. Asterisks show significant values. "Doctors Guess" refers for an attempt by the EEGer to guess whether the record represented a control or exposed subject. Significant probability levels are indicated by asterisks.

(3) Visual Inspection of Sleep EEG

Table 7 shows the results of the C x E and C x M analyses of the all night sleep EEG records. No significant differences are seen for the four stages of slow wave sleep or total sleep time. On the other hand, absolute time spent in REM is significantly increased for the CxM analysis (.02 level) and the % time in REM sleep increased for the CxE ($P < .02$) and C x M ($P < .01$) analysis.

The results of the all nite sleep study show that the entire exposed group showed an increase in REM sleep and that this was of slightly greater magnitude for the maximum exposure group.

Discussion

The results of the monkey study indicate that a single symptomatic exposure or a series of subclinical exposures to the OP, sarin, can alter the frequency spectrum of the EEG for up to 1 year. The major effect was a persistent increase in beta activity (13-50Hz) seen mainly in the occipital-temperal (O-T) EEG during the states "awake in darkness" and "drowsy". The small dose schedule also showed an increase in the frontal-central (F-C) derivation.

The beta increases in the large-dose animals were evident at 24 hr post-drug and persisted. We feel that the large-dose beta increase is the result of a persistence of acute actions of the drugs. Following the slow iv injection of sarin at the large-dose level, the EEG decreased in voltage and increased in frequency producing a record of almost

TABLE 7

HUMAN SLEEP EEG

	ABSOLUTE TIME					PERCENT TIME				
	REM	Stage 1	Stage 2	Stage 3	Stage 4	REM	Stage 1	Stage 2	Stage 3	Stage 4
C	37.740	41.233	199.225	20.311	5.510	11.834	14.760	64.703	6.681	1.665
E	45.913	44.916	182.432	17.478	5.680	15.203	15.173	61.288	5.773	1.808
CxE	(T).1	---	---	---	---	U.015* T.015*	---	---	---	---
M	51.655	51.019	193.406	20.301	6.649	15.747	15.129	60.144	6.277	1.703
CxM	U.02* T.02*	---	---	---	---	U.015* T.01*	---	---	---	---

Results of analysis of all night sleep EEG on human subjects. C, E, CxE and CxM have the same meaning as in table 6. Time in each phase of sleep is shown in minutes and again as percent of total sleep time. Comparison was by t-test and U-test. Asterisks show significant levels. Non-significant results are indicated by a horizontal dash.

continuous low-voltage beta activity. This was followed by irregular bursts of high-voltage sharp waves in the 6-10 Hz range intermixed with the high-frequency background. Finally, there was almost continuous seizure activity consisting of runs of spikes, polyspikes and spike-waves separated by periods of relative electrical silence. This "burst-suppression" pattern continued for about 1 hr in most animals and then was replaced by a post-ictal record consisting of high-voltage slow waves in the 1-3 Hz range. By 24 hr, the EEG was still dominated by slow-wave activity, but the initial high frequency beta activity was still present (see Fig 2). Our results would indicate, therefore, that of the EEG alterations acutely produced by a single convulsive dose of sarin, spike activity and slow waves are spontaneously reversible, but the increased beta activity persists for up to a year.

A significant result of this study was the effect produced by multiple small-dose exposures. The dose levels used in these treatment schedules did not produce the overt signs of poisoning seen with the single, large doses. However, the multiple small-dose exposures produced the same type and degree of EEG alteration as the single large-dose exposures. Indeed, for sarin, multiple small doses appeared to produce more change than the large dose. This is surprising in view of the fact that the multiple small-dose group showed no overt clinical manifestations related to drug administration, whereas the single large-dose group suffered a severe CNS insult (prolonged electrical convulsions). It is to be noted that the total dose received by the multiple small-dose group was roughly twice that of the single-dose group. Thus long-term EEG changes may be more a function of cumulative drug dose than of clinical manifestations at time of exposure. Note, also, that the small dose schedule had a more widespread long term effect, as it involved both O-T and F-C derivations; the large dose schedule affected only the O-T derivation.

The results of the human study demonstrate that workers with histories of exposure to sarin have waking and sleep EEGs that differ significantly from those of workers of no exposure history. These differences were:

1. Spectral analysis - increased beta activity in the temporal and occipital-central leads of the maximum exposure group ($P \leq 0.05$).
2. Visual reading of standard EEG - decreased background voltage ($P \leq 0.015$), and decreased amounts of Alpha ($P \leq 0.05$).
3. Sleep EEG - increased amounts of REM sleep ($P \leq 0.01-0.02$)

Spectral analysis failed to show statistically significant differences between the control and exposure (CxE) groups, but did demonstrate highly significant differences between the control and the maximum exposure (CxM) group. These differences tended to cluster in one frequency range (beta, 12-30 Hz) and were found in both the temporal (T3-T5) and occipital-central (OZ-CZ) electrode montages. This is the very same frequency band and brain area where persistent EEG changes were found for monkeys one year after sarin exposure.

Statistical analyses of the visual EEG readings showed significantly decreased background voltage and lower alpha amplitudes, without a significant increase in background delta or theta slowing. This suggested general background "desynchronization."

All night sleep EEGs demonstrated significant increases in the percent of time in REM sleep for both experimental ($P \leq 0.02$) and maximum ($P \leq 0.01$) groups. Absolute REM time was also increased but just for the maximum group. No other sleep stage, taken as percent or absolute, showed significant differences. The fact that an increase in REM was significant for the exposure group only when expressed as a percent suggested that there must have been a slight decrease in slow wave sleep for individuals in this group, not enough to be significant by itself, but enough to increase the percent REM. Percent REM for all three of our groups (Including the controls) fell below published normals for middle aged men.[14] This may be due to either or both of the following reasons. First, REM is usually decreased during the first night of sleep in a laboratory situation (first night effect).[14] Second, most REM tends to occur in the final third of a night's sleep. All our subjects were awakened at 0530, approximately 30 to 45 minutes before their usual waking time. Thus, most of this lost sleep may have been REM. Our results confirm the finding of Metcalf and Holmes, who also reported an increase of REM sleep in subjects with histories of OP exposure.[10]

What is the significance of these findings? De-synchronization in general may be a sign of simple decrease in neuronal synchronization (normal alert state), or it may signify functional neuronal depression (hypothermia, drug effect), or neuronal depopulation (aging, dementia).[15] Following intravenous administration of a convulsive dose of Sarin, monkey EEGs show a brief period of desynchronization prior to the onset of background slowing and seizure discharges. At subconvulsive dose levels, desynchronization may persist for some time.

Increased beta activity may be due to any number of factors. Beta is increased by drowsiness, sleep deprivation, and certain drugs. As can be seen from Figure 2, a marked increase in the beta content of monkey EEG follows intravenous sarin injection. Lower, subclinical doses of sarin produce longer periods of increased beta. This increase in beta is quite consistent with the findings of desynchronization on visual inspection. By definition, desynchronization usually includes an increase in fast activity (beta) in addition to a decrease in background voltage and a decrease in slow (delta, theta) activity. Fink has reviewed the relationship between EEG and behavioral effects of drugs.[16] Significantly, many of the chronic symptoms reported for insecticide exposure are found to be acutely associated with EEG patterns of increased beta activity and desynchronization. These include irritability, anxiety, memory defects and thought disorder. Drugs producing acute patterns of increased beta activity comparable to the chronic pattern induced by sarin include some of the most potent psychoactive agents such as amphetamine, bemegride, dimethyltryptamine, LSD-25, mescaline and psilocybin.

The significance of increased amounts of REM sleep is unclear. Of the 20 or more pathological states associated with clinical derangements of sleep, as described by Williams, almost all involved no change or decrease in REM.[14] In a review of REM sleep pharmacology, Hartman listed over 20 drugs causing a depression in REM (including barbiturates, common tranquilizers, mood elevators, alcohol, and amphetamine), but only four causing an increase in REM (Reserpine, L-tryptophane, 5-OH-tryptophane, and LSD).[17] Thus, it is unusual to find drugs that increase REM. A significant exception to this are OP compounds. One patient examined by the authors following accidental exposure to the OP, soman (GD), demonstrated a strikingly desynchronized EEG and complaints of a dream-like state of visual hallucinations associated with an inability to fall deeper asleep (Unpublished Observations). Flurries of rapid eye movements were noted during the 1st day after exposure period, although no EEGs were obtained.

There have been several reports recently of a cholinergic link involved in REM sleep mechanisms.[18-21] Administration of cholinesterase inhibitors elicited REM sleep in both intact animals and brain stem preparations.[20] It has been postulated that cholinergic mechanisms are involved in REM initiation.[18,19]

What are the pharmacological mechanisms underlying the persistent effect of OP exposure? Acute exposure to OP compounds produces pro-found CNS effects ranging from subjective changes in behavior at low doses, to convulsions at higher doses. Furthermore, prolonged lower level exposures may produce a psychiatric syndrome resembling schizophrenia.[1-6] All these are believed to be secondary to reductions in CNS cholinesterase levels. Accordingly, recovery is believed to parallel return of CNS cholinesterase to normal. On the other hand, monkeys exposed to sarin showed increased temporal lobe beta one year after sarin exposure.[11] Studies of tissue cholinesterase in monkeys usually demonstrate a return to normal within three months of exposure.[22] Furthermore, the abnormalities demonstrated in the human study were found in subjects with normal plasma and red blood cell cholinesterase levels. Thus, evidence of CNS abnormality attributable to OP compounds can be found in monkey and man at a time when tissue cholinesterase levels are probably within normal limits.

One explanation may be that the low tissue cholinesterase levels at the time of exposure induce long-term changes in synaptic morphology or biochemical organization which do not completely reverse when tissue cholinesterase levels return to normal. Evidence for or against this is lacking.

Another explanation may be that sarin has actions beside that of a potent anticholinesterase. Similar compounds, triorthocresylphosphate (TOCP) or difluorphosphate (DFP) are known to produce peripheral neuropathies of delayed onset. Sarin can also produce such disturbances in peripheral nerves but only in the chicken, not in primates.[23,24] Some of the simpler OP compounds also produce central lesions of delayed onset similar to the peripheral lesions. Sarin is not thought to do this, but complete pathological studies are not available. The monkeys which showed persistent EEG changes following sarin exposure were not studied in this regard. Thus, one might speculate that the persistent CNS effects in primates may not be related to anticholinesterase properties, but to some direct lesion of axons or myelin.

In either case, since the long-term findings (desynchronization, increased beta, and increased REM) are also seen as part of the acute exposure syndrome, the pathogenesis of the long-term effects probably represents an unexpected and possibly irreversible extension of the primary drug effect.

Regardless of the pathogenic mechanisms, results of the current study confirm the ability of OP compounds to induce persistent abnormalities in the electrical activity of the primate brain. Whether these long-term neurophysiological changes can be directly related to reported behavioral abnormalities is not known. On the other hand, the long-term behavioral changes and long-term electrophysiological abnormalities provide parallel evidence that OP exposure, even at subclinical levels, can produce long term change in the brain function of monkey and man.

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DR. F. COULSON: Dr. Bradley, I certainly appreciated very much your presentation, and I think you've made several important points. You've made it very clear, that there is a dose level, with all of these compounds, where there is no effect on the central or peripheral nervous system. Would you expect then that you could have a transitional dose response until you reach an effect you can see as a pathologic change.

DR. W. A. BRADLEY: Yes. I think there probably is a no-effect level. But with a lot of these compounds, using repeated dosing, you get an accumulative effect. This has particularly been seen with TOCP, as shown by the early work done by Smith and his co-workers. They definitely showed there was a no-effect level to a single dose, but when several lower doses were given, delayed neurotoxicity could be produced. There was an even lower dose, quite low down, where even with repeated dosing they could get no effect. So the things to look for are: one, the effect after a single dose, and two, the effect after repeated dosing. I think with most of these you probably would find eventually that there is a no-effect level, certainly as far as histological signs go.

DR. A. COELHO: This question is directed to Dr. Duffy. I have a few very short questions dealing with your control group. First of all, were your animals,your Macaca mulatta, feral or laboratory raised and how large with respect to body weight were they?

DR. F. H. DUFFY: They were laboratory raised about three to four kilograms and they received a dose of 5 milligrams per kilogram.

DR. COELHO: That's a fairly young juvenile. Were there both males and females included:

DR. F. H. DUFFY: Yes.

DR. A. COELHO: Were your controls real controls, or were they basically reference groups in terms of EEG values? In other words, did they receive sham injections?

DR. F. H. DUFFY: Yes, they were studied before and after sham injections. They were also paralyzed and respired at the time of sham drug administrations.

DR. N. ALDRIDGE: This certainly isn't my field and I'm not sure whether I understood what you told us. What is the variation in the controls of the monkey experiments over a very long period of time when measurements are taken daily? And the second question, if you give monkeys Sarin, where you get the changes in the REM sleep pattern? Now does this follow with respect to time after dosage?

DR. F. H. DUFFY: Sarin usually produces an increase in REM sleep lasting about 48 hours following a single dose, then the increase tails off. There may be compensatory decrease in REM sleep for a few days thereafter. Regarding the controls, we did not take just one measure. We studied them for six separate recording sessions before the drug. The one year value was based upon, six or eight separate recording sessions. If there had been wide variation, this would have reflected itself as a large variance, which would have rendered the T-test or any statistical comparison, non-valid. The results were quite consistent, internally, among themselves. Also, any change that came up in both the controls and the experimentals would not have been significant.

DR. J. P. FRAWLEY: Under what set of circumstances were these 79 individuals exposed to Sarin? Was it laboratory exposure? Occupational exposure? Human volunteer experiment?

DR. F. H. DUFFY: These were accidental industrial exposures, and their exposure was documented and treated in a clinical facility. They were not volunteers. This is not a part of the Army's medical volunteer program. These were industrial accidents.

DR. J. P. FRAWLEY: So in the year since Sarin exposure, because of their occupation they might have been accidentally exposed to other chemicals.

DR. F. H. DUFFY: They were very carefully screened, for exposure to other toxic substances. We intentionally screened out any of those who had histories of exposure to chlorinated hydrocarbon or exposures to substances at the nearby Shell Chemical Company plant. No subjects had documented exposures to any compound in the year prior to this study. Our screening procedure left us with only a small number of subjects with uncomplicated Sarin exposure. But of course, they could have had gardening as their hobby, for all we know. This, of course, would apply to the control group, too.

DR. J. P. FRAWLEY: But were they working in a chemical environment?

DR. F. H. DUFFY: Everyone was! They were all employed in the same installation where Sarin was maintained. But the exposed group had histories of exposure and of access. The controls were never in a position where they would be exposed, barring a major catastrophe.

DR. J. GEHRICK: I'm interested in your experience with not being able to classify these people as normal or abnormal. Yet, you found with other computer processing of the data, there were some things that were different. Would you care to reflect on what that means, in terms of clinical abnormality or possible future clinical abnormalities?

DR. F. H. DUFFY: Well, just to clarify again what we found on the human study, a population of exposed individuals differed from a population of control individuals. I'm sure if we had taken 79 people off the street in Denver, and 79 people off the street, living at sea level, we would have found differences. But we could not have attributed them to any one factor. So the fact that we see differences in the two groups is significant insofar only as we were carefully able to control other important variables. We tried to think of every possible item. We felt that the only difference between the control and exposure groups was the history of exposure to Sarin. The results simply say the populations are different. It is our presumption that this is due to Sarin exposure.

I couldn't take a single individual and say anything by visual inspection of his EEG recording or by looking at his spectrum. Now, it's very hard to go from a population study to an individual basis. But because we could do it with the monkeys and because we had the clue that there was something there on the population level, we thought it would then be interesting to then get much more involved in this question. In other words, perhaps the only reason you can't take an individual and say he belongs to the control or exposured group is due to the limitation of your brain to make very subtle, multitudinous correlations. There are very many factors to consider. Maybe the mind cannot appreciate trends that are really there. That is where pattern recognition can be helpful.

Here is how it is done. You develop 20 factors for each individual of two populations. You feed data on all these individuals and their associated factors into a computer and you define which of the people are exposed, and which are controls. The computer comes back and says, for example, that only parameters one, five, nine and thirteen are necessary to differentiate the two groups. So, then you measure those factors for each individual, and you keep refining your parameters until you discover, rather empirically, by interacting with the recognition program, which parameters are of no use and which are good. It didn't take very long before you were able, with only a ten percent error, to take an individual and say from which group he came,

DR. J. GEHRICK: What do the things that you found, mainly increased REM and increased fast activity in the exposed group, mean to you as a clinician?

DR. F. H. DUFFY: That is a very hard question to answer. Low background amplitude, increased fast activity and increased REM time are the classic signs of a drug effect. I believe our study suggests that individuals exposed to Sarin may be under its pharmacological influence for a much longer time than previously anticipated. However, one cannot say that these individuals are brain damaged. Rather, our study seems to provide support for material in the literature which suggests a long term effect following organophosphate exposure. Maybe there is something to the behavioral complaints that people come up with. Neither that nor any other specific correlation was made in this study.

DR. W. N. ALDRIDGE: I'm very interested in pursuing this question a bit more because of the implications of what you say. Do I understand you that your technique involves taking these two populations of humans, one of which has a history of exposure to Sarin, and the other has a history of working in the same factory with no exposure -- that is the situation?

DR. F. H. DUFFY: That's correct.

DR. W. N. ALDRIDGE: You then have put into the computer your records, and have been enabled by selection to get the situation that the groups are always different.

DR. F. H. DUFFY: That's correct.

DR. W. N. ALDRIDGE: Now, what is the next stage? Do you then have to go out and get another group of totally non-exposed people and show that they always go into the control? You see, you're starting with a kind of fixed population, two groups that you've selected by one means, where you defined the parameters. But, they're restricted to these groups. Do you now have to go out and take somebody who works in a different occupational environment and see that they always correspond to the controls?

DR. F. H. DUFFY: You see, we come to this question again. It isn't enough to simply show a difference between two populations. This has been a fault with many statistical studies. You have to be sure that the only thing that these populations differ by is this measure and a critical factor in their history. For example, the population that we're concerned with is largely Mexican-American, in the age group between forty and sixty, peaking at 45, living at 5,000 foot altitude. It would not be correct to compare this population to a white middle class population of both sexes living at sea level. Any statistically significant results we might find could be attributed to a variable host of factors.

DR. F. H. DUFFY: Just living an upper middle class life and not being exposed to toxins of the slum may in fact be a more potent factor in causing differences than exposure to Sarin. So, yes, if we had a larger population unexposed to Sarin, but with everything else being the same, that would make a much better control group but great care should be taken to ensure a useful control population. We have saved another group of exposed individuals, to retest this whole hypothesis. But we're saving them for inclusion in the pattern recognition study.

DR. W. J. HAYES: I have two totally unrelated questions; Were these exposures associated with illness? And, if so, how much or what degree of illness? Has this exact kind of study you've made, ever been done with people who might be expected to be concerned, for whatever reason, about their situation? In other words, these people who had been exposed the year before knew perfectly well they'd been exposed and the controls knew perfectly well they were off in another part and were no more exposed there, than you and I are here. So there had to be some awareness on the parts of the individuals, even though they were removed and exposed.

DR. F. H. DUFFY: I don't know how one could control that. This approach has been used for other EEG based studies and other population studies. Yes, there's no question that anxiety over the results of this study might have affected the results. However, most of the individuals did not seem to demonstrate anxiety, but were rather annoyed at having to have this break in their routine. They were simply told that a new regulation required EEG studies of all employees.

To be entered in the study they had to have clinical signs of exposure and more than a 25% reduction in their baseline cholinesterase levels. This included everything from severe emergencies to treat-and-go-home situations. We did not have a large enough population to look at the changes versus the degree of exposure to quantify it and look for trends.

DR. H. FEINMAN: In your animal studies, I believe there were some statistically significant differences in the alpha, beta or delta or theta wave patterns between treated and untreated animals that were still demonstrable a year after exposure. Was any necropsy done at the time, to determine whether these differences, on histopathological examination, correlated with any demonstrated pathological lesions.

DR. F. DUFFY: There were no consistent changes in delta, alpha and theta, only beta. There were just as many controls who would turn up with increased or decreased alpha as experimentals that turned up with decreases. In other words, there were random changes as well as the specific ones. Post mortums were done on all animals but special stains to look for demyelination were not used. There were no visible histological lesions found with routine stains.

QUESTION: Did you imply that REM sleep is not a good thing?

DR. F. H. DUFFY: Not at all, it's necessary in the appropriate amount.

QUESTION: I would hope that anything that could prolong REM sleep might be good for us. It's the best part of sleeping. It's when we make all our nice dreams, have hallucinations about all sorts of things, and also have persecutions. I didn't want the audience to get the impression that we had interfered with this good part of sleeping.

DR. F. H. DUFFY: I agree, REM sleep is the most enjoyable part. It is to be recalled however that during REM sleep you have the highest incidence of heart attacks and epileptic seizures, if these are the underlying disease. REM sleep is necessary and desirable in appropriate quantities. Too much or too little REM might be harmful and might also signal underlying disease.

DR. V. SIM: I might state that the animals also underwent extensive psychological behavioral testing and were followed over a period of two years with all the parameters that were available at that particular time. There was nothing that we were able to pick up in the behavioral patterns or the psychometric testing that gave us any lead as to what we saw, as Dr. Duffy has discussed.

DR. F. H. DUFFY: These animals were tested at 24 hours after exposure and they showed nothing on delayed performance test using Wisconsin General Test Apparatus. We didn't think to test them a year later because we didn't think we would be getting into the question of delayed onset.

ORGANOPHOSPHATE EXPOSURE FROM INDUSTRIAL USAGE, ELECTRONEUROMYOGRAPHY IN OCCUPATIONAL MEDICAL SUPERVISION OF EXPOSED WORKERS

K. W. Jager, M.D.

In the first part of my paper I would like to deal specifically with the problems of monitoring and controlling the exposure of industrial workers to potentially neurotoxic compounds, which is a responsibility of the industrial medical doctor. In the second part I propose to review the literature concerned with neurotoxic effects, especially in relation to organophosphate insecticides.

Electromyography (EMG) and electroneuromyography (ENMG) provide sensitive and objective methods of detecting impairment of nerve and muscle function. With these methods a.o. the conduction velocity of nerves and the action-potential of muscles resulting from a controlled stimulus on the nerve can be measured. Abnormal reaction patterns of the muscle may be recognized and it is possible to measure reflex times.

In the past, EMG and ENMG were not suitable for health supervision in occupationally exposed workers because needle electrodes had to be introduced in the tissues near the nerve and in the muscle. More recently newer methods have been developed in which surface electrodes can be used and which give accurate and reproducible results.[1] In general, a supramaximal stimulus - this is a stimulus to the nerve which will evoke a maximum reaction in all the muscle fibres - is given via electrodes placed on the skin over the ulnar nerve, and the action potential and action pattern of the adductor muscle of the thumb is recorded.

This method was first used in the supervision of occupationally exposed organophosphate workers, when the late Professor Andrew Wilson of the University of Liverpool and Dr. D. V. Roberts of the same University introduced it in the Industrial Medical Department of Shell's chemical works in Rotterdam in The Netherlands. Groups of organochlorine and organophosphate workers as well as a control group were then examined and significant deviations from the norm were found in approximately 50% of the workers exposed to organophosphate insecticides.[2] This was the more remarkable, because regular determination of the blood cholinesterase activity in this group of workers, when compared with the results of their individual pre-exposure value, only sporadically indicated possible overexposure.

Since that time, a close scientific cooperation has been established between Dr. Roberts of Liverpool and Dr. Ottevanger, who now supervises the health of our pesticide workers, with the result that our organophosphate insecticide workers have been checked regularly by means of ENMG in addition to their normal medical examination and the regular determination of blood cholinesterase activities.

On further periodical supervisory examinations we continued to find in some of the exposed workers the same deviation from the norm, which is qualitatively related to the intensity and the duration of the exposure, neither of which can be measured directly. Initially, an effect of base materials and intermediates could not be excluded, but later, similar, though less pronounced changes were found in sprayers in the field, who were exposed to the endproduct[3,4] only.

The changes found are the following:

1. A decrease in the voltage of the action potential of the muscle. In non-exposed controls most values would be between 11 and 12 mV, in the exposed workers the range would be wider and values could occasionally be as low as 4 mV.
2. A change in the pattern of the recorded electromyogram, indicating that not all muscle fibers react at the same time to the supramaximal stimulation.
3. A decrease in the conduction velocity of the nerve fibers.

In our organophosphate workers, all of whom have a full medical examination at least once a year, we have never seen any signs and symptoms or overt disease which could be related to the above findings. One should, however, realize, that the complaints one might expect to hear would be very unspecific, such as fatigue, weakness, etc.

Further results of the continued work of the same group have been,[204,205] or will shortly be published.[206,207]

The following actions are presently taken when workers show the above-mentioned aberrations from the norm. The exposure situation is closely scrutinized and where possible improved. Workers who show progressively decreasing voltages on successive examinations would be transferred to other work where they have no further contact with organophosphate insecticides, until the reason for the effects has been detected and corrected.

The workers in the plant are fully aware of the nature of this monitoring procedure and welcome it. Experience over the last few years has shown, that the rapid feed-back of information derived from ENMG-studies helps to maintain a high standard of industrial hygiene, and as a result of this the incidence and severity of the abnormalities may be kept as low as possible.

Transferred workers are followed up, and so far all abnormalities have returned to normal. This generally takes a few weeks or months, but sometimes up to a year.

This slow recovery and the changes found are in accordance with a diagnosis of subclinical neuropathy. Neuropathy, no matter how slight, must be regarded as a more direct parameter of human health than a quickly reversible cholinesterase depression. The evaluation of the significance of neuropathic changes is primarily a medical function.

Recently, very thorough reviews on peripheral neuropathy caused by chemical agents[5,6,7] have been published, as well as reviews of the application of neurophysiological methods[5,210] to occupational medicine.

It is well known, of course, that members of the group of organophosphorus compounds can cause cholinesterase depression or may have neurotoxic effects, and in some cases may do both. Currently one is inclined to believe that members of this group of compounds in principle have both capacities, but that they vary in the expression of these effects.

In view of the well established neurotoxic effects of TOCP and some other O.P.-compounds, all organophosphate insecticides are tested for possible neurotoxicity by a laboratory test on hens. However, we do not really know how relevant the "hentest" is as regards the potential of long term occupational exposure as a possible cause of neuropathy.

From the medico-toxicological as well as from animal experimentation literature complete studies covering all aspects of clinical signs and symptoms, neurophysiology and pathology are scarce. They are, however, an increasing number of papers relating to the subject which mention, separately, clinical disease, neurophysiological abnormalities or pathological changes, such as peripheral neuropathy, myopatny, visual disturbances, cochlear and vestibular effects and behavioral changes in relation to intentional or accidental, and sometimes occupational exposure to a wide range of organophosphate insecticides and carbamates.[9-72,132-171, 211-216] Clear cases of peripheral neuropathy following intentional or accidental overexposure have been published. Cases of clinical paralysis in man have been described,[164-167] even with "safe" products, such as malathion.[164] With a few compounds animal experiments have shown that both partial demyelination and an effect on the axon cylinders occur. At times, these changes have even progressed to necrosis in the muscle.

The neuropathy in experimental animals and man is by no means limited to organophosphate insecticides. It can also be produced by a wide range of chemicals, such as

- TOCP[73-81, 172-174]
- lead[82-86, 175-177]
- acrylamide[87-88, 178-179]
- carbondisulphide[89, 180-183]
- ethanol,
- methylbutylketone and its metabolite 2,5-hexanedione[90-92, 184]

- trichloroethylene,[185]
- hydrocarbon solvents such as hexane, heptane and low-boiling gasoline fractions[93-112, 186]
- triethyltin[187,188]
- methylmercury[113-116, 189-193]
- arsenic compounds[194,195]
- hexachlorophene[117, 118, 196-198]
- dithiocarbamates[119,120]
- diphenyl[121,199]
- thalidomide[122,200,201]

but also by therapeutic agents, such as,

- diazepam[123]
- chloroquine,[124] and
- dapsone[125]

Diabetes,[126] diphtheria,[127] allergic reactions,[128] as well as compression and/or crushing of nerves[129] and vibration by vibrating tools[130,202,203] may cause a similar neuropathy. Deficiency diseases, certain virus diseases and idiopathic polyneuritis[208,209] could be added to this list.

There may be some qualitative differences in the effects caused by these chemicals and other factors: some affect the sensory system more than motor nerves, some have more central effects than peripheral ones. The end-result, however, appears to be similar for all compounds mentioned, although the quantitative aspects are of course quite different.

The list of substances and factors mentioned is by no means complete, nor is the list of references at the end of this paper.

In this group of chemicals lead is of special interest because in this case cause and effect are very well documented and quantified, both from animal experiments and from human clinical experience.[82-85] It is known that in the past many cases of lead-paralysis have occurred. Moreover, the ENMG changes found with lead closely resemble our data with organophosphate insecticide workers. From animal experiments we know that long-term oral exposure to lead may result in segmental demyelination and axonal degeneration with typical ENMG-changes.[82] The same ENMG-changes are found in workers with known heavy lead exposure and a history of past lead poisoning.[84] Similar ENMG-changes may be present, albeit to a lesser extent in lead-workers who have never exceeded acceptable lead levels in blood and who have never had a lead-poisoning, nor any signs and symptoms.[85]

With the organophosphate insecticides we appear to have a similar situation: the parameter commonly used in the supervision of the workers, the blood cholinesterase determination, is a measure for exposure. ENMG-changes, however, may occur unrelated to depressions in cholinesterase activity and certainly there is a phase-difference between the two, for it may take days or weeks for ENMG-changes to appear following over-exposure, and it may take months to recover from it. As with lead, these changes may develop in a situation which was believed to be "under control" using parameters of exposure only. The WHO in a recent Technical Report on "Early detection of health impairment in occupational exposure to health hazards" also refers to this finding.[131] The Pesticide Subcommittee of the Permanent Commission on Occupational Health in its Workshop on Cholinesterase in Cambridge in September 1975[217] came to a similar conclusion.

The significance of these findings in relation to health is at present still unknown. Our experience in man[218] and other evidence from the literature indicate, that there is probably a no-effect level. Data obtained on control-groups demonstrate that the general population is not affected.

Our data on organophosphate workers indicate that the changes are reversible, but the literature shows that in more advanced stages which are accompanied by clinical signs and symptoms this may not always be so. Reversibility possibly depends upon both the magnitude and the duration of exposure. Of course, with many organophosphate insecticides, exposure is limited by the acute toxicity of the product, except in those cases where tolerance has developed and the usual muscarinic warning signs and symptoms may be diminished or absent.

We do not yet know at what point the individual or statistical trend becomes of significance to health. We feel, however, that it is prudent to assume, that if exposures, severe enough to cause such changes, are allowed to continue unchecked, it might in the end lead to clinical signs and symptoms.

Cholinesterase determinations and electroneuromyography both have their own place in the medical supervision of organophosphate workers. Which one gives the first indication of overexposure probably depends on the timing of the test in relation to the exposure and the degree and duration of exposure. ENMG-changes may be completely unrelated to cholinesterase depression. Another esterase may be involved, or there may be a completely different mechanism, as is possibly in the case of other chemicals causin ENMG-changes.

All this of course leads to the conclusion that further work is required:

1. To elucidate the mechanism by which organophosphates cause these ENMG-changes,
2. on the dose-response relation as related to the time factor,
3. on the reversibility of the effect.
4. No-effect-levels have to be determined in subchronic and long term animal tests, using ENMG techniques, as well as light- and electronmicroscopy of nerve tissues.

Although there is reason for caution and concern, a final decision on the significance and acceptability of the ENMG-changes mentioned will only be possible when some of these questions have been answered.

At the present time ENMG-monitoring has a practical value in that it may be used in industrial hygiene supervision, as a check on the efficiency of protective and safety measures, and as another source of information on which the industrial medical director can base his clinical judgement.

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DR. M. K. JOHNSON: I was impressed by your telling us all the details. I think many people who, such as myself, don't know too much about industrial exposure would be grateful if all companies were as straightforward in declaring what they found and how they dealt with it.

I would like to ask whether if the route of exposure, were dermal rather than systemic, might that mean the possible effects could arise from a local inhibition of acetylcholinesterase without much effect being seen on blood cholinesterase? Also, is it possible within the whole group of organophosphoruous esters that your company produces, whether you can, in any sense, correlate bigger or smaller effects with individual compounds? In other words, if you can do sort of a mini-structure/activity survey. And, if one were to think that perhaps one is looking for a target, and you consider whether these are effects on acetylcholinesterase, or on neurotoxic esterase, or on some other target altogether, one means of coming to some decision might be to consider workers exposed to carbamates. Because, as my hypothesis stands and as the observations go, it would seem impossible to produce a typical organophosphate neuropathy with a carbamate. But you could still get chronic anti-cholinesterase effects.

DR. K. JAGER: Thank you, Dr. Johnson. First, with respect to the route of exposure, it is of course very difficult to tell what is the major route of exposure. In a general way, we have the feeling that the main route of exposure, at least in our organophosphate plant, is dermal. Maybe with the exception of those few cases where we manufactured DDVP. In those cases there may have been to a certain extent an inhalation exposure.

With regard to the oral route, -- well, you cannot completely exclude it, of course. But we have taken all sorts of measures to exclude that route. In our opinion, it's mainly the dermal route of exposure.

As to the various compounds, I must say that we have seen this effect with all the organophosphates which we manufacture. We do not manufacture them all at the same time. Rather it's done blockwise. So we can really look at it and say, well, now it's this substance and now it's that substance.

We have had an occasion where we had a developmental carbamate, which was not manufactured on an industrial scale. But on the laboratory scale, where, in fact, we also had these effects. And it had a very prolonged effect.

With the carbamates, we thought that it would be impossible to supervise employees in a proper way with the normal cholinesterase determination method which we were using the Michel method, and that we would have to use the Ph-stat method. Using electroneuromyography, we found changes, which we checked regularly. It took months and months for the recordings to become normal again. They became normal, but only after a long time.

DR. R. CLYNE: I'm rather intrigued by your mention of the fact that there were some people who allegedly developed paralysis as a result of exposure to malathion. Could you elaborate on that and give us some documentation? I think Dr. Coulston, many years ago in British Guinea, had an opportunity to study people who in attempting suicide had horrendous exposure to malathion. It was my understanding that there was no evidence for paralysis in any of these people.

DR. K. JAGER: Well, I do not know how good this publication is. It's a fairly old publication in the American Journal of Medicine in 1958 cited in my list of references. It was a massive overexposure! A physician who used malathion in his garden thought that spraying with 25% concentrate every day would be better than doing it in a normal way. So he went on and on and he got a paralysis and he died in the end.

DR. R. CLYNE: I can only say that with the experience we've had with that compound over many years, close to 25 right now, including people who had horrendous exposure in the holds of liberty ships in the James River, where they had no protective clothing of any kind and where they sprayed and were sprayed with, in many instances, the concentrate, for up to four months, the people suffered no effects at all. There was no depression of cholinesterase nor effects noted with any gross test. I must confess we did not do ENMGs or EMGs on them. But, there were no findings at all in the nervous system such as you allude to.

DR. K. JAGER: I would agree with you that this is a very exceptional case. Reading through the case histories it's a very exceptional case. I would not normally expect this to occur with malathion. On the other hand, when you say that even in massive prolonged exposure we do not see cholinesterase depression, this may be right. But it could be that if you look at an ENMG, you would find changes. As I have mentioned, and it has been mentioned earlier today, it could well be that very toxic, or highly toxic organophosphates or carbamates, by the very effect of their high toxicity are used more carefully, and you would see less ENMG changes with those. Whereas, with the less toxic materials, you're less careful, you get a higher exposure, and you may see more ENMG effect. Working on the assumption that it's an unrelated effect.

DR. J. SANBORN: This comment will be brief, since Dr. Johnson has already reported on the neurotoxicity of desbromoleptophos, a photodegradation product of leptophos. We have found the same results at the same levels and Dr. Roy Fukuto has repeated and confirmed these findings. With leptophos oxon, we found the same thing. In addition, desmethyl leptophos, at 200 milligrams per kilogram, does not seem to be neurotoxic. Dr. Fukuto has also tested it at 500 mg/kg, and it's not neurotoxic.

One further note, not related to neurotoxicity; chickens given 25 milligrams desbromoeptophos in a single oral dose in gelatin capsules are still excreting material in the eggs, if they still lay eggs, at about half a part per million in the eggs. This indicates the stability of this photo-degradation product, which is also a contaminant of technical leptophos.

DR. J. E. CASIDA: What is the acute LD_{50} value for desbromo leptophos?

DR. J. SANBORN: I have no idea but it's not particularly insecticidal as far as I know.

DR. J. E. CASIDA: In other words, is the acute toxicity: delayed neurotoxicity ratio for desbromoleptophos similar to that for leptophos.

DR. M. K. JOHNSON: No! It's much easier to produce the neurotoxic syndrome with much less cholinergic response. I give atropine, and I give P-2S, but it's very easy to show neurotoxicity with desbromoleptophos and less easy with leptophos.

DR. J. E. CASIDA: I would like to introduce Dr. Abou-Donia who was the first to report in the literature the delayed neurotoxicity of leptophos. I'm sure his comments will be of considerable interest.

DR. M. ABOU-DONIA

I don't think that I can add any more to the information that we just heard from the excellent speakers today. I have only a few minutes to present two year's worth of work. However, you might consider my presentation as an application or an example of the lessons that were taught today on delayed neurotoxicity.

Chemically leptophos (Phosvel)^R is 0-(4-bromo-2,5-dichlorophenyl) 0-methyl phosphonothioate. My attention was first focused on leptophos in 1971, when it was implicated in the poisoning of about 1,300 water buffalos in the Nile Delta. The clinical condition was permanent ataxia followed by paralysis and death. Two other pesticides were sprayed in that area: Cyolane^R [2-(diethoxyphosphinylimino)-1,3 dithiolane and Hinosan^R [0-ethyl SS-diphenyl phosphorodithioate]. The signs of delayed neurotoxicity observed in the field were reproduced experimentally in water buffalos fed forage sprayed only with leptophos. Subsequently, we established that leptophos caused delayed neurotoxic effects in fowls 10-13 days after the oral administration, intubation, of a single dose in corn oil (Abou-Donia, et al. 1974). A dose of 160 mg/kg, resulted in "no effect" while [1] higher doses (180-3,000 mg/kg) caused delayed neurotoxic effect in some cases. The most noticeable sign was a disturbance in the control of movement of the legs with a change in the gait. In each bird the earliest sign was a weaving of the gait, apparently secondary to leg unsteadiness. This evolved into a gross ataxia. As time passed, the signs progressed: legs sprawling out in front, inability to stretch the legs, difficulties in moving the legs, inability to stand, and down on the hocks most of the time. An occasional dragging of the legs was observed. The weakness of the legs was manifested by the unwillingness of the birds to walk. When made to do so, the dragging of the legs was very prominent. Once ataxia appeared, the bird's decline was rapid, and paralysis occurred 15-23 days after the administration of leptophos. A rapid irregular head tremor was present in some birds and was especially pronounced in paralyzed birds. After paralysis, all birds showed respiratory and swallowing difficulties. Recovery was never observed in any bird having developed ataxia. The severity of the neurotoxic signs and the number of birds that developed the condition were dose-dependent; but no matter how great the dose, the latent period before onset of neurotoxicity was never less than 8 days.

Later, in collaboration with Dr. S. H. Preissig, we carried out an investigation into the nature of the neurological lesions produced in hens administered three neurotoxic doses of leptophos; 200, 400 and 800 mg/kg in corn oil and gelatin capsules (Abou-Donia and Preissig, 1976).

Signs of neurological dysfunction were present 8-14 days after oral administration of a single dose of leptophos. In general, the onset of ataxia and paralysis was not markedly affected by the insecticide carrier.

All leptophos-treated birds lost weight. During the latent period, between the administration of leptophos and the onset of ataxia, there was virtually no change in body weight. Significant loss occurred in the hens between the onset of ataxia and paralysis. The loss in weight sharply increased during the time between paralysis and death or sacrifice. At the beginning of the experiment, the hens acted normally and consumed amounts of water and feed comparable to control birds. Anorexia was noted after the onset of ataxia, which increased as signs of neurotoxicity progressed to paralysis. Paralyzed hens gradually stopped eating. The number and weight of eggs was decreased in most of the treated hens.

Figure 1 shows hematoxylin and eosin (H&E) with Luxol fast blue (LFB) stained longitudinal sections of sciatic nerve exhibiting numerous foci of swollen myelin sheaths and fragments myelin (x650). These changes were present in the majority of birds.

In Figure 2 the loss of myelin is seen in the anterior columns of sections from the lumbar spinal cord.

Figure 3 is a diagram representing the pattern of degeneration of myelin and axons (Black area) in the central nervous system of hens administered leptophos. The lateral ascending tracts are involved in the medulla. These lateral as well as the ascending posterior tracts are damaged in the cervical cord. Only early degeneration in the anterior descending tracts is seen in the thoracic cord. The lumbar cord shows severe involvement of this anterior pathway.

H&E sections of muscles from the majority of hens were not significantly different from controls. NADH diaphorase stain revealed clearing of the central portion of a large proportion of muscle fibers; this represented dissolution mitochondria, sarcoplasmic reticulum, and other subcellular organelles. Wing muscle from chicken 4 showed several foci of small, angulated atrophic fibers.

Initially leptophos appeared to inhibit red blood cell acetyl cholinesterase significantly. In general, there was a dose dependent

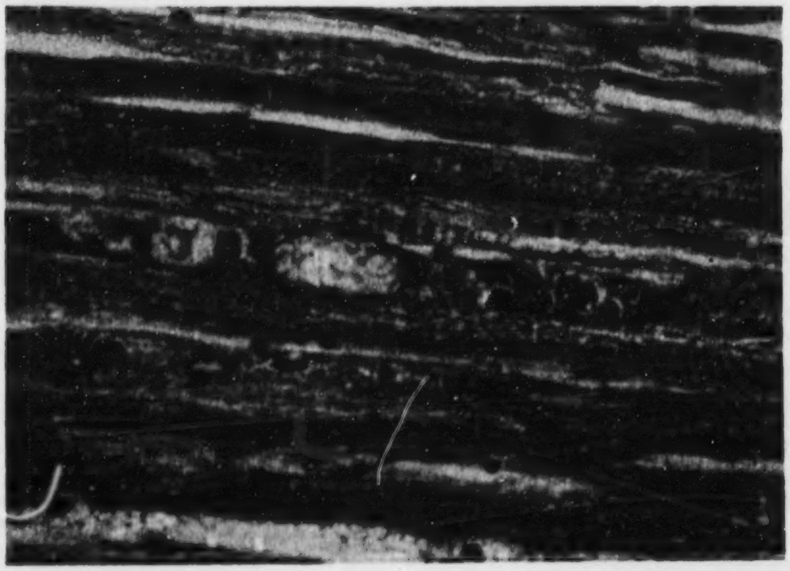

FIGURE 1.

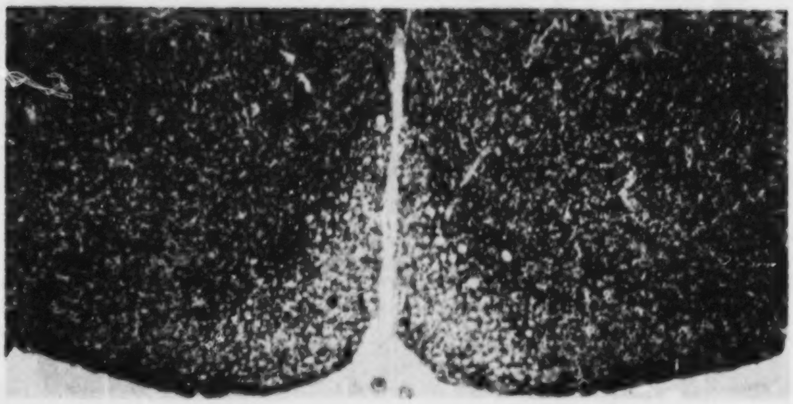

FIGURE 2.

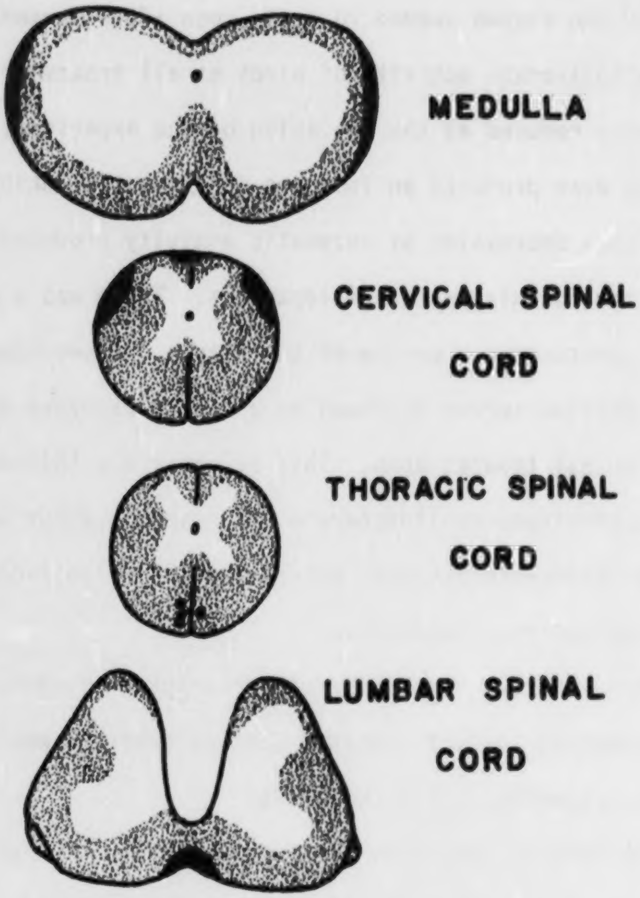
MEDULLA
CERVICAL SPINAL CORD
THORACIC SPINAL CORD
LUMBAR SPINAL CORD

FIGURE 3.

inhibition of red blood cell acetylcholinesterase. The inhibition was greatest 1 day after the administration of the compound. Spontaneous recovery of the enzyme seemed to begin soon after maximal inhibition. Plasma cholinesterase activity of birds at all treatment levels was significantly reduced at the beginning of the experiment. Again, an increase in dose produced an increase in enzymatic inhibition. There was an initial depression of enzymatic activity produced by all doses one day after administration of leptophos. There was a considerable amount of spontaneous recovery of the enzyme. A secondary depression of plasma cholinesterase followed by a second recovery of this enzyme was noted in all treated hens. This recovery was followed by a third depression of plasma cholinesterase beginning at about day 20-27. Plasma cholinesterase activity values remained significantly lower than those of the control, thereafter.

Our latest study has confirmed our original findings that the delayed neurotoxic effect reported for leptophos resembles that of other neurotoxic organophosphorus compounds.

The ability of long term low-dose administration of leptophos to produce delayed neurotoxicity in hens was investigated. Table 1 shows that 6 dose levels of leptophos were administered daily: 0.5, 1, 2.5, 5, 10 and 20 mg/kg in gelatin capsules. Three hens were given empty gelatin capsules and served as controls. The dosing continued until ataxia developed but no longer than 60 days.

Administration of small subneurotoxic doses of leptophos ranging between 1 and 20 mg/kg could build up and cause neurotoxic effects. At

Table 1

Sequence of intoxication, onset of clinical signs and histological changes in tissues from hens fed a daily single dose of leptophos[a]

Hen	Dose	Days of Administration				Duration of Intoxication	Histological Changes	
		Ataxia	Paralysis		Sacrifice		Sciatic Nerve	Spinal Cord
			Onset	Recovery				
A	20.00	29	39	--	85[b]	56	+	++
B	20.00	22	26	--	50	28	+	++
C	20.00	29	35	--	77[b]	48	NE	NE
D	10.00	39	58	102	108	69	-	+
E	10.00	39	56	112	216	177	-	+
F	10.00	32	61	102	216	184	-	+
G	5.00	59	--	--	216	157	-	+/-
H	5.00	58	--	--	59[b]	1	NE	NE
I	5.00	59	--	--	123[b]	64	-	+/-
J	2.50	25	--	--	103	78	+/-	+/-
K	2.50	65	--	--	103	38	-	-
L	2.50	65	--	--	103	38	NE	NE
M	1.00	68	--	--	160	92	NE	NE
N	1.00	62	--	--	160	98	NE	NE
O	1.00	62	68	73	160	98	-	-
P	0.50	--	--	--	78	--	NE	NE
Q	0.50	--	--	--	78	--	NE	NE
R	0.50	--	--	--	78	--	-	-

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[a] The following abbreviations were used: NE, tissues not examined; -, changes absent; +/-, changes equivocal; +, changes present; ++, severe degeneration.

[b] Hen died shortly before dissection.

these levels the dose was almost completely cumulative. The dose of 1 mg/kg was a "threshold dose" below which no effects were produced. A "no effect" daily oral dose of 0.5 mg/kg was found, which produced no signs of ataxia, no histological changes and no marked variation in the weight and number of eggs laid during 60 days of feeding. Duration of administration of subneurotoxic doses of leptophos varied inversely with the size of the daily dose. Also, the "total dose" that caused ataxia was proportional to the size of the daily ingested dose. However, this was not merely a cumulative effect, since at lower daily doses there was a decrease in the total dose required to produce ataxia. This finding may be attributed to a more efficient absorption and/or metabolism of divided doses than of a single dose. This is certainly true with larger doses, where one single dose of 160 mg/kg of leptophos was ineffective, but 60 daily doses of 1 mg/kg were neurotoxic. Hens fed a daily dose of 20 mg/kg of leptophos developed marked ataxia and paralysis with no recovery observed. When smaller daily doses were fed, the condition of the hens deteriorated during the first 2 weeks after onset of ataxia, then remained stationary for a period of several weeks before gradually beginning to improve. The pattern of myelin and axon degeneration in the spinal cord and sciatic nerve in the long-term feeding of small doses to hens was identical to that produced by a single dose of leptophos and to that found in the classical picture of organophosphorus neurotoxicity.

A pharmacokinetic profile of [^{14}C] phenyl leptophos was determined in laying hens following a single oral dose of 75 mg/kg (1.0 μCi). Table 2 shows that most of the radioactivity was excreted into the urine

Table 2

Cumulative Percentage Recovery of ^{14}C Radioactivity From Hens Given A Single 50mg/kg Oral Dose ($1\mu C_i$) of ^{14}C-phenyl Leptophos

Days	Expired Air	Excreta	Egg Albumen	Egg Yolk	Tissues	Contents of G.I.	Total
½	0.05	28.55	0.41	0.24	14.58	47.24	91.06
2	0.16	70.94	1.24	0.78	14.51	5.98	93.61
4	0.40	77.16	1.79	1.34	14.29	0.52	95.51
8	1.03	79.75	2.26	2.05	10.61	0.15	96.05
20	1.25	86.47	3.40	2.54	6.21	0.13	100.00

and feces. After 20 days, the total radioactivity recovered in the combined urinary-fecal excretion was 86.47% of the administered dose as compared to 2.54% in the egg yolk. Radioactivity was least excreted in the expired CO_2. The half-life for the disappearance of radioactivity from the bird's body following the administration of [^{14}C] leptophos was 11.55 days which is much longer than biologic half-life of most organophosphorus pesticides.

Our studies have demonstrated that leptophos causes delayed neurotoxicity in chickens which resembles that of other neurotoxic organophosphorus compounds. They have also shown that small subneurotoxic doses of leptophos can build up to cause neurotoxic effects in hens. Since it is assumed that a compound showing such activity might produce the same effect in man, and in view of its long biologic half-life, leptophos requires careful consideration before widespread use.

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PESTICIDE-INDUCED DELAYED NEUROTOXICITY: POISON CONTROL OR MEDICAL ASPECTS

John Doull, M.D., Ph.D.

Introduction

Emergency room physicians and others concerned with the management of acute pesticide poisoning were first alerted to the possibility that OP insecticides might produce a "Ginger-Jake" like paralysis in 1952 by the clinical note of Bidstrup and Hunter[1] and their subsequent report[2] on three cases of Mipafox poisoning. Two of the three cases described by Bidstrup, Bonnell and Beckett[2] were severely poisoned and subsequently developed paralysis whereas the third case exhibited less severe acute symptoms and no delayed effects. In the first case, a 28 year old female research chemist, the acute episode lasted for about four days and she was then essentially symptom-free until leg weakness developed about ten days later. During the next two weeks, her symptoms progressed and on readmission she was found to have flaccid paralysis in both legs, loss of reflexes and impairment of both the upper limb and trunk musculature. There did not appear to be any CNS or sensory involvement although the patient later exhibited some transient sensory changes. Motor function returned gradually in this patient over the next six months with the legs being the last to recover. She also exhibited severe muscle wasting in the hands and feet and there was still slight evidence of this effect at eleven months after exposure. The time course, signs and symptoms in the second patient, a 39 year old male chemical process worker, were similar to those of the first patient although his acute poisoning episode did not appear to be as severe as in the first patient. By ten months after the initial exposure, the second patient was able to return to work but he continued to experience "cramp-like" pain in the right foot and calf muscles. Although the case reports in this paper suggest that both of these patients were overdosed with atopine during the acute episode, there is certainly nothing in the way of symptoms, laboratory values or clinical course which would distinguish the acute poisoning episode in these two cases from that seen with other organic phosphate insecticides.

Since there were no other clinical reports of neuromuscular paralysis following OP insecticide exposure with the possible exception of a German greenhouse worker exposed to parathion[3], it seemed likely that these two cases represented a specific effect of Mipafox rather than a general effect of organic phosphate insecticide poisoning. This position was supported by the results of chronic animal studies with OMPA and E605 (parathion) which had been carried out in our laboratories in Chicago[4,5] and in the laboratories of Barnes and Denz at Carshalton[6]. However, in subsequent studies, which were stimulated by the Mipafox paralysis report, Barnes and Denz[7] were able to produce weakness, ataxia and paralysis in chickens and weakness in rabbits with Mipafox. They also demonstrated that similar effects could be produced with DFP which confirmed and extended previous observations made by Modell _et al._[8], Hunt and Riker[9] and by Koelle and Gilman[10,11]. In evaluating their studies, Barnes and Denz[7] pointed out that although the effects and lesions produced in chickens by exposure to these two fluorophosphates were similar to those which they observed following tri-o-cresyl phosphate (TOCP) exposure, the effects of Mipafox, DFP and TOCP could be distinguished from the effects of other cholinesterase inhibitors by the marked species dependency, the relative insensitivity of young animals, the specificity of the neurologic damage and the similarity of the lesions to those seen in cases of vitamin B_1 deficiency. Thus, even at this early research stage in 1953, it was evident that several significant advances had already occurred. First, the potential hazard of delayed neuromuscular paralysis as a result of OP insecticide exposure was recognized and there was concern as to whether this represented a generalized clinical problem or was an isolated response to fluorophosphates. Second, it was clear that although the response was highly species-variable, it appeared that chickens would provide a satisfactory model test system for detecting and investigating the response. Third, it was evident that since cholinesterase inhibition could not be directly implicated as the mechanism responsible for the neuromuscular paralysis in either human or animal studies, it would be necessary to look for other common or independent mechanisms to explain these effects.

During the next twenty years most of the research effort in this area was focused on the same three problems: compound specificity or structural-activity relationships, species variability and mechanism of action. Numerous reviews of the progress which has been made in each of these areas have appeared in recent years[12-18] and the current status of each of these problems has been considered in the previous presentations at this conference. The purpose of this presentation is to consider some of the poison control or medical aspects of organic phosphate induced paralysis with particular emphasis on the three traditional aspects of poison control: first, prevention; second, diagnosis or detection; and third, treatment or management.

Before doing this, however, I would like to comment briefly on some of the research which was carried out at the University of Chicago Toxicity Laboratory under the direction of the late Kenneth P. DuBois. Although we were more concerned in these programs with the problem of characterizing the structure-activity relationships responsible for the acute toxicity of the organic phosphate insecticides, we did look for histologic evidence of peripheral nerve damage in each of our chronic dog studies and, initially at least, in some of the chronic rat studies. Included in these studies were such compounds as Guthion (azinphos-methyl), Coral (coumaphos), Systox (demeton), Meta-Systox-R (oxydemeton-methyl), DiSyston (disulfoton), Tiguvon (fenthion), Dasanit (fensulfonthion), Dylox (trichlorfon), Chlorthion and DEF (tributyl phosphorothioate). During this period we also carried out chronic feeding studies with several carbamates such as Baygon (propoxur) and Matacil (aminocarb) and with various Chemagro fungicides such as Morestan (oxythioquinox), Eradex (thioquinox) and Dexon (fenaminosulf). We did not see delayed paralysis,in any of the animals from these studies and we were also unable to detect histologic evidence of damage in the dog sciatic nerve sections. I should point out, however, that in the majority of these studies we did not look at the distal end of the sciatic nerve and that several of the compounds which we tested did turn out to be proneurotoxic when tested later on in chickens using the Barnes and Denz protocol[19]. In addition to those agents which were subjected to chronic toxicity studies, DuBois and his colleagues also investigated the toxicity of many other phosphates and carbamates, and in many cases chickens were included in the list of test species. Since these were regular oral LD_{50}

tests with a two week observation period, one would not have expected them to detect paralysis except perhaps with agents having low toxicity and a very potent neurotoxic effect. During our studies on BFP or dimefox, for example, we found it to be ten to fifteen times as toxic as Mipafox to rats, mice, guinea pigs and dogs[20] and DuBois suggested[21] on the basis of this toxicity and its weak cholinesterase activity that it would not be neurotoxic. Subsequent studies by Davies et al.[22] demonstrated neurotoxicity not only with dimefox but also with the ethyl derivative which was even more toxic than dimefox. At this stage, the "state of the art" in predicting neurotoxicity on the basis of structural relationships was still pretty much at what Aldridge describes as the "guess and test" level[23,24]. It is really only in relatively recent years that progress in understanding the biotransformation of these agents and additional testing has enabled predictive toxicology based on structural-activity relationships to become reality[25].

Prevention of Pesticide-Induced Delayed Neurotoxicity

As a first step in evaluating the poison control or medical aspects of pesticide-induced delayed neurotoxicity, it is appropriate to consider the real and potential magnitude of the problem. This subject has been reviewed by Hayes[26] in 1969 and again by Namba in 1971[27,28]. If we restrict our evaluation to the incidence of delayed neurotoxicity from organic phosphates and exclude the neurotoxic effects of other pesticides, such as the dithiocarbamates, and metal pesticides, such as arsenic, lead and mercury, it is clear that delayed neurotoxicity is a rare and rather inconsistent occurrence. In his review of the previous literature, Namba[27] found only seven cases where he felt that neuropathy may have resulted from OP insecticide exposure. In analyzing these and other possible cases, Namba et al.[28] point out that two of the three parathion cases exhibited muscle weakness and decreased tendon reflexes for only two weeks[3], that both of the cases described by Petty[29] were exposed to mixtures of insecticides (DDT in both and lead arsenate in one) and that in only one of the four cases where malathion has been reported to cause neurotoxicity[30,31,32,33] was there a truly delayed effect. Namba also comments[27] that he has not found neuropathy among patients with acute poisoning by organophophorus insecticides and cites the absence of neuropathy and myopathy in the five year follow-up survey of 398 OP insecticide workers by Kovarik and Sercle[34] and in the survey by Davignon et al.[35] of pesticide exposure in 441 apple growers. There is an additional report of delayed neurotoxicity following parathion exposure[36] but in this case the major sequalae (partial blindness and focal epilepsy) were central rather than peripheral.

In an effort to check on the current poison control situation regarding neurotoxic effects from pesticides, I went over the records of the 1300 poison control inquiries which we received last year. Twenty-seven of these involved pesticides and all but two were subsequently seen either in our emergency room or elsewhere. There were six cases of organic phosphate poisoning which required hospitalization (3 malathion, 2 parathion and 1 DDVP), and although muscle weakness and fasciculation was present during the acute phase in several of these patients, none of them exhibited any sign of cranial nerve disfunction, peripheral neuropathy or persistant muscle weakness when examined within a week or two of their exposure. I also checked with Dr. Vernon Green, the head of the Toxicology Unit at the

Children's Mercy Hospital in Kansas City and with Dr. Barry Rumack, the director of the Rocky Mountain Poison Center at Denver General Hospital. Dr. Rumack estimates that they treat over 100 cases of moderate to severe organic phosphate insecticide poisoning each year but have seen no delayed neuromuscular paralysis in any of these patients. With similar reports from poison control surveys in Nebraska[37], Arizona[38] and Florida[39], it is tempting to simply agree with Namba's evaluation[28] that "Although studies have indicated neurotoxicity in some organophosphate insecticides in certain species of experimental animals, persistent neuropathy in man by poisoning due to commercially available organophosphate insecticides seems exceptionally rare, considering the large number of incidents of acute poisoning. Manifestations were variable among reported patients and not comparable to the results in experimental animals." Although I agree generally with this position, I do have two problems. The first is that during the past few years there have appeared a number of Russian and Rumanian papers reporting delayed paralysis or toxic polyneuritis with trichlorofon primarily but also with other organic phosphate insecticides[40-51], and the second is that I think it is dangerous to adopt generalizations about any class of pesticides which is as chemically diverse as the organic phosphate insecticides.

In a very real sense, the emergency room physician is at the end of the line in that he usually encounters these problems long after the basic research and decision-making processes have taken place and often the kind of practical information which he needs is not available. When we see a report, for example, which indicates that leptophos causes neurotoxicity in chickens[52], it would be helpful to know whether leptophos is simply another OP insecticide which produces paralysis in hens but does not cause similar effects in man or whether leptophos is, in fact, one that we really have to worry about. Clinical toxicologists are also committed to the idea that animal studies are predictive for man but all of us would feel more secure about the OP delayed paralysis problem if the registration requirements for these agents included some type of low dose human study coupled with a sensitive and sophisticated electromyographic search for neuromuscular effects. At the very least, we need a mechanism by which the ER physician can readily obtain data on any unusual health problems observed in the workers engaged in the production, formulation and use of such products. Optimally, this information should be a part of the regular poison control information systems such as the National Clearing House cards or the Poisindex microfiche.

One other suggestion which has been made repeatedly in meetings of the American Association of Poison Control Centers and the American Academy of Clinical Toxicology is that regulatory agencies should exert more pressure on pesticide users to use the least toxic agent which is capable of doing the job. This recommendation is based on the fact that most of our cases of serious OP poisoning are due to parathion or methyl parathion and that other countries like Japan and Denmark have reduced the incidence of OP poisoning by legislation and various types of campaigns to encourage users to select the less toxic alternatives even though they may cost more money.

Diagnosis and Treatment of Pesticide-Induced Delayed Paralysis

The symptoms and time course of the paralysis seen in the "Ginger-Jake" episode of 1931[51,54] were similar to those reported from Switzerland in 1940[55], from Morocco in 1959[56] and from Fiji in 1969[57]. In addition, most cases of Apiol poisoning (an extract of parsley dissolved in TOCP and used as an abortifacient) also exhibited latency, flaccid symmetrical paralysis of the limbs with minimal sensory and CNS effects. When these effects are compared with the reports on the cases of delayed paralysis following OP exposure, the only difference which seems significant, aside from the immense difference in incidence, is that the recovery seems to be more rapid and to be more likely to occur in the OP cases. Although there have been attempts to classify TOCP as an upper motor neuron poison, it is now clear that both TOCP and the paralysis producing organo-phosphate insecticides affect initially and most severely the lower motor neurons although spastic paralysis and other signs of upper motor neuron damage may occur subsequently.

Cavanaugh[17] has described the lesion as a "dying-back" process in the peripheral nerves and suggests that this is metabolic in origin, and Johnson[12] has provided convincing biochemical evidence as to the nature of the actual target for initiating the changes. The possibility that many of the toxic agents which produce motor or sensory disfunction, and perhaps even some of those that produce lesions higher in the nervous system, may be involved in a common or at least related type of injury, is exciting since it may result in new approaches to prevention and therapy not only for the organic phosphates but for other toxic agents where the problems are more immediate.

Finally, in regard to the therapy of phosphate paralysis, it is evident that the only real hope for preventing or minimizing the delayed sequela is early treatment. Most of the therapeutic measures previously tried (atopine, oximes, vitamin B_1, cortisone, etc.) do not appear to be be particularly effective[58,27]. However, Johnson[15,59] has recently shown that the prior injection of certain carbamyl, sulphonyl, and phosphinyl esters can protect hens against the neurotoxic effects of DFP or TOCP and these findings would appear to offer some therapeutic possibilities. It would be interesting, for example, to see if these agents are as effective against the neurotoxicity of agents producing neuropathy of rapid onset (abate, azinophos, carbophenothion, coumaphos, crotoxyphos, crufomate, dicapthon, dioxathion, disulfoton, EPN, ethion, fenthion, malathion, methyl parathion, methyl carbophenothion, phorate, and ronnel) as they are against the agents which produce delayed neuropathy like DFP, Mipafox, DEF, and Dursban. From a poison control viewpoint, it would also be helpful to know whether any of these agents are effective when given after the phosphate exposure and, if so, the length of time during which they are effective.

In conclusion, I think it is fair to summarize the poison control concerns about the problem of phosphate-induced delayed paralysis as more of a sword of Damocles than as a serious current problem. This is not so for other toxic agents or even for other pesticides[60] and I think that the most encouraging message that I will carry away from this conference is that progress involving the problem of phosphate-paralysis may ultimately improve our ability to prevent the neurotoxic effects of a whole variety of toxic agents.

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DR. M. K. JOHNSON: Perhaps I could make a comment concerning the possibility of contrast, between cresylphosphate and pesticide poisoning in humans. The severe case, Mary Whittaker, who had the substantial dosing with mipafox, has not recovered significantly. There is considerable muscle wasting. She can travel about 100 yards, with effort and with the aid of her crutches. So, I think it's rather unlikely that a severe case of neuropathy from a pesticide would turn out to be much different from cresyl phosphate neuropathy.

As regards human studies, we are setting up a very simple exercise in our laboratory, to compare the general in vitro behavior of human neurotoxic esterase with hen neurotoxic esterase. This will tell us nothing about the problems of different metabolic conversions, excretion, or indeed the different sensitivity of the human nervous system to insult at the enzyme active site. But it will at least tell us whether basically the active site is similar in the two species. That I think is probably about as far as we shall be able to go.

As regards the chlorophos cases, I did make the suggestion that the Russian cases might be due to an impurity. Certainly the specimens of chlorophos I've seen from various parts of Europe vary in their general appearance and purity. However, in the laboratory, they do not appear to differ in response when compared with pure trichlorophon. Now I think I should say, a propos trichlorophon, the same as I said about DDVP. It is only under the most heroic conditions that I have ever been able to get an ataxic hen with trichlorophon treatment. This was on double dosing with huge quantities, two or three days apart, coupled with tremendous doses of atropine, and oximes, and very, very ill birds. The Russian sample that I managed to get, which is only one out of many perhaps which are circulating, was very dirty. But in biological activity, it seemed about the same. When I mentioned impurity, what I had in mind was that if one happened to use an impure preparation of methanol in the synthesis, one which contained ethanol to some degree, that would increase the neurotoxic potential very dramatically. One factory might do it occasionally and that was a possibility.

Regarding antidotal treatment of the rapid onset paralysis, I haven't tried it. I have never produced that lesion in the laboratory. I have never tried. And it was put to me quite forcefully last night that I shouldn't make the assertion that it's different without showing that it's different. However, let me state, the reasons why I think it's different. First of all that with EPN oxon you can very easily get the delayed neurotoxic effect without any rapid onset paralysis. I find it hard to believe that the rapid onset paralysis is mediated by anything other than a chronic release of the oxon. Secondly, as I read the published papers, the brain cholinesterase was inhibited for a very long time, more than two weeks after EPN and right down near the base line. I therefore assumed that rapid onset paralysis is in fact chronic cholinergic stimulation.

DR. J. DOULL: I appreciate that additional word on the one case. Is that true also for the second case?

DR. M. K. JOHNSON: I'm honestly not sure! I've met Mary Whittaker. I haven't met the other person. I've got the impression that with that one, it wasn't a very severe case by comparison.

DR. J. DOULL: Actually what that really does then for me is to distinguish the mipafox cases even further from what has been reported subsequently in the pesticide literature.

DR. F. H. DUFFY: As a clinician, I have to interject the speculation that the neuropathic process may be more common that we think, because in reality we rely upon overt clinical signs and symptoms to bring this to our attention. Suppose someone comes with an acute cholinergic crisis, a drop in cholinesterase, or a history of exposure and is at risk. He's usually studied very intensely at this point and then sent home. Now, if signs of a subtle disorder of peripheral nerve conduction should develop, chances are very likely that this would not result in symptoms of such severity for the patient to present himself to a physician for reexamination -- I use as a model -- the spontaneously occurring neuropathies, diabetic, alcoholic, tabetic, that we see very day in the clinic. People will walk in with the complaint of a headache, or something else, and you'll discover very pronounced neuropathic changes. Patients may be totally unaware of this.

You can also look at people in their twenties who are at risk for diabetes, for example, and discover lower nerve conduction velocities, missing ankle jerks or signs of neuropathy of which they are totally unaware. As a neurologist, I pick this up all the time.

So I suspect that if you did a study in time on someone exposed to these compounds, you might see changes in the following 3-10 days especially if an objective test of nerve conduction velocity were used. This kind of approach to the problem is totally lacking, except in a few very well equipped laboratories.

What this means is you would probably need to do base line nerve conduction studies on people at risk, maybe cortical conduction velocities with evoked potential as well. Next one would study them immediately after exposure and then serially for two weeks to see if there is any change. And if there is a change, whether it comes back to normal.

Most of the studies have been "one shot". Is this person within the range of normal? But you don't know whether he moved from high normal to low normal. And whether or not subtle changes are seen across a large group of exposures. So I suspect that there's a lot of epidemiological work that needs to be done.

Point two is that the term neuropathy has been used consistently here, but there's involvement in -- even in the chick -- in the spinal cord. And the correct term for this is a radiculopathy or myelopathy. So, if you do literature research on the subject, you should really look at radiculopathy neuropathy, and/or myelopathy.

DR. J. DOULL: I did and I apologize for using the less precise terminology.

I might say, in regard to the possibility that some of the people we have treated in the emergency room and sent home have in fact developed paralysis that we are not aware of. If they were seen in our hospital and they return to any clinical service in our medical center, it is more than likely that we would know of it through our consult mechanism. Since this has not occurred, I think I can say reliably that none of our cases of organic phosphate poisoning have developed delayed paralysis.

I would agree with you about the need for more epidemiology studies in this area. There is a fair amount of literature from California and elsewhere concerning central nervous system changes after organic phosphate exposure. Some of these studies reported effects such as muscle weakness, fatigue, irritability, etc., which may involve peripheral nerve disease, or may be due to central effects. I think we need to look closer at both and I think we need to begin to pay more attention to some of these low level chronic complaints that are mentioned in the literature.

DR. W. N. ALDRIDGE: I'm interested in Dr. Doull's statement that we have had no real problems with pesticides in this field, and I would agree with him. But I think perhaps it would be useful to think if we can understand why this is so. The fact is that most pesticides have been designed, or in retrospect they appear to have been designed primarily as inhibitors of cholinesterase. And it happens that the structure/activity relationships, such as we can discern them, are the inverse of one another for cholinesterase inhibition and the production of this lesion, i.e., the production of this lesion depends on long, rather large groups attached to the phosphorus. In contrast, for the cholinesterase, and therefore for the pesticides, by and large small groups are better. Of course, there are some exceptions, e.g. EPN.

So I think this is perhaps largely the explanation for the difference between the tri-ortho-cresyl phosphate (TOCP) series and the pesticide field. In terms of clinical recovery as well, I think there are other factors to consider. Dr. Doull mentioned, of course, the question of re-education. There are other features which may be important which are compound determined. One is the particular relationship between the peripheral and central damage. This appears to vary although detailed information is sparse with the compound. It may vary with the species and it may vary also with the dose, i.e. whether it's long term or short term. And of course, if the damage is peripheral, then recovery is likely to be almost complete.

The other thing which I would like to emphasize here, is what Dr. Barnes kept hammering home. That we must pay attention if we get worried about compounds to humans whom we know have had substantially more exposure, than to the general population, i.e., occupationally exposed people. We must pay very much attention to this and make as many measurements as we can on these people.

DR. J. DOULL: I would like to support Dr. Aldridge's comment on occupationally exposed workers. We need to have a mechanism whereby industry could funnel information on all of the atypical reactions that have occurred in their workers during the manufacture of each product. Oftentimes that is exactly the kind of information that the emergency room physician needs to manage difficult cases. I don't know what the mechanism should be to accomplish this, but I've heard the same suggestion from a lot of emergency room people.

DR. W. N. ALDRIDGE: I think it is quite clear that from the acute poisoning point of view, organophosphorus pesticides really aren't much of a problem. Poisoning is almost always due to inhibition of cholinesterase. There are practically no examples, except for mipafox, which was not a commercial pesticide, of an acute episode of poisoning by pesticide leading to delayed neurotoxicity. But nevertheless, the problem still stays in our mind as to whether if there are repeated episodes of poisoning, cumulative damage is produced. I hope that from this conference, a variety of people will go away with the message that we now have a few more parameters to measure. We can measure phosphorylation of a particular protein, and in properly designed experimental situations, in the hen, where this question can be properly explored.

DR. J. DOULL: I should have mentioned previously that I did talk to a number of people from industries that are engaged in the manufacture of phosphates, to ask them if they had had any neuromuscular-worker health problems related to pesticide workers. I was unable to uncover any.

DR. F. COULSTON: I would like to point out that we must separate the accidental poisoning episodes relating to the factory or occupationally exposed individual from one of the real hazards, the presence of pesticide residues in foods. I would like to comment on the annual joint meetings of the FAO/WHO Working Party and Expert Committee on pesticide residues in food. Annually this group reviews data on new and old OP compounds that have been assessing the toxicological significance of residues in and on food. It is the trend, year after year now, to study some of these in man. And not only do they consider and evaluate animal data, but invariably a 30-day test on man is considered. These are often administered at doses based upon the residues or tolerances that might be recommended to be in the food at the time of consumption.

Now, what I'm driving towards is that heroic doses are all acceptable for experimental purposes, to try and develop rationale for a mechanism of action. I would even doubt this mechanism means much if you have to use atropine and 2-PAM, and artificial ways to keep, in this case, the chicken, alive. What is important is that studies be established, both in animals and man, at reasonable doses or reasonable factors of the intended use for that particular chemical.

Now, for example, it was at the beginning of the meeting that it was pointed out that the recent 1975 FAO/WHO Joint Meeting on Pesticide Residues did indeed recommend a temporary tolerance for leptophos. In doing this, they took into account not only the positive chicken data, but the fact that there was no effect in any other species, for example, the dog, a susceptible species which was studied over a 2-year interval. A comparative toxicology view was a necessity in this instance. Now, giving leptophos a temporary tolerance and a temporary ADI entailed allowing an enormous safety factor. Not as large as has been used in some other compounds, but one 2,000 times the observed no effect level. I would like to emphasize again, what is learned inside the factory and by accidental means is important. But we should always keep in mind how these compounds are to be used.

DR. J. DOULL: Thank you, Dr. Coulston. I think you and Dr. Duffy and Dr. Aldridge and all of us are saying something important to the EPA. I hope so.

DR. M. K. JOHNSON: Could I just say again I agree very much with what Dr. Coulston has said concerning these ones which have been shown to be neurotoxic only under most heroic conditions. However, if I could just raise a plea, I think the laboratory testing is tremendously important to make us aware on a numerical sort of basis. If we can get a graded response on sample biochemical testing, we are much more aware of that with which we are dealing. And we must face the fact that although food tolerances and residues may be important, they are very much a maybe. We're not quite sure whether anybody has really suffered much from eating food from plants treated with pesticides of any sort. But if there's an accident, it would be a great pity to work like mad to get somebody through the cholinergic symptoms and then to find that they're paralyzed for the rest of their lives. We are dealing with a more or less irreversible damage in really severe cases. And therefore ought to focus rather carefully on accidental and severe exposures.

DR. G. ZWEIG: I just would like to ask Dr. Coulston a point of information. What was that two thousand safety factor based upon? On the no effect level of what symptom?

DR. F. COULSTON: The conclusions were based on the fact that in the chicken a dose response curve could be established and a no-effect level could be observed with leptophos. This is not realized by many people, and I must say I wasn't quite sure of it. But that was one of the cardinal principals involved. So, with a no-effect level in a chicken, and with no-effect levels noted in many other species of animals that were studied, it was possible to apply 2,000 times safety factor, to the lowest no-effect level noted.

DR. G. ZWEIG: But was it cholinesterase or was it delayed neurotoxicity?

DR. F. COULSTON: The FAO/WHO Joint Meeting suggested the rat and the dog as sensitive species for evaluating cholinesterase inhibition. The actual no-effect level was based on plasma and red blood cell cholinesterase depression values.

With the hen, we based a neurotoxicity no-effect level on data showing an acute dose of 50 mg/kg in hen. The doses for the rat and dog were 30 and 60 ppm respectively. These doses are equivalent to 1.5 and 2.1 mg/kg body weight for the rat and dog respectively. From this an ADI of 0.001 mg/kg was estimated which is a 2000-fold safety factor.

The point to make is that, having seen the no-effect level based on the most sensitive species with respect to cholinesterase depression and applying a large safety factor there is a much greater margin of safety than just basing an ADI on delayed neuro-toxicity dosage related to the hen.

DR. R. J. RICHARDSON: Dr. M. K. Johnson's discovery of neurotoxic esterase should have great practical benefit in neurotoxicity testing, and I hope intelligent use of this assay will be made in assessing the neurotoxic hazard of new compounds. However, I can see a potentially greater benefit arising out of continued studies of this unusual neuronal protein. When clinicians speak of 'neuropathy' or indeed of a wide variety of spontaneous neurological disorders, they are speaking of diseases with unknown etiologies and unknown methods of cure. With neurotoxic OP compounds, we have a chemical means to produce neurological disease, and a defined molecular target, 'neurotoxic esterase', as the triggering point of the neuropathological process. It would seem that a thorough study of neurotoxic esterase and its role in chemically-induced neuropathy should prove extremely rewarding in the study of neurological disease and in the understanding of long-term maintenance processes in the normal nervous system. Our group at Michigan hopes to continue along these lines, in collaboration with Dr. Johnson and his group in Carshalton.

TEST PROTOCOLS AND LIMITATIONS FOR DETECTION OF NEUROTOXICITY

John P. Frawley, Ph.D.

At this point in the Conference, it is unnecessary for me to provide an introduction in the form of relevant research reported in the literature. Other speakers have already thoroughly reviewed the most significant work and have identified the area of concern which brings us together.

However, I do believe that you are entitled to an introduction of a different type, because the data I will present are over twenty years old. I believe these data are highly relevant to the selection of animal models to evaluate the chronic neurotoxic effects from organic phosphate pesticides and provided the bases for an FDA policy 20 years ago on evaluating the safety of organic phosphate pesticide tolerances - a policy which I suspect has long been forgotten.

By way of explanation, let me give you the ancient history of these data. For a period of time in the late 40's and 50's, I worked for the Division of Pharmacology of FDA, which many of us refer to as Dr. Arnold Lehman's old Division. I was assistant to Dr. Garth Fitzhugh who was Head of the Chronic Toxicity Branch. We became interested in the problem of myelin degeneration as a result of the poisoning cases in Great Britain by Mipafox reported by Lesley Bidstrup in 1953. Our interest was not related to the potential health hazard to the manufacturing worker or the agricultural applicator, which in those days were the responsibility of the Public Health Service and Department of Agriculture. Our concern was focused on the potential of the spray residue on food to produce myelin degeneration from long-term or chronic ingestion. To evaluate this potential, we conducted a series of long-term chicken and pigeon feeding studies on a series of organic phosphate pesticides. I gave a preliminary report on these studies in the Spring of 1956 at the Society of Pharmacology meeting and the abstract of that paper was published in the Federation Proceedings. We terminated our studies later in 1956 and I left the Food and Drug in December 1956. I cannot be certain why FDA or I did not publish the results of the studies but I suspect it was the difficulty in those days to get unsensational toxicological data published. In the meanwhile, everyone who had participated in anyway in these studies or even had knowledge that they had been conducted left FDA via retirement or for other reasons. To make matters worse, the responsibility for regulation of pesticides was transferred from FDA and apparently the policy on suitable protocols was also lost in the shuffle.

When I was asked to present a paper at this symposium, I delayed acceptance until I started digging to locate more of the data than that contained in the brief 1956 abstract. Fortunately, I had taken copies of much of the data, including pathology reports with me when I left FDA. Presumably, I did this with the intent of someday writing a report. Dr. Robert E. Zwickey, now with Merck Institute of Therapeutic Research, was the pathologist who worked with me on this project and I wish to acknowledge his contributions. Also collaborating with me on these studies were Henry Fuyat, biologist and Dr. J. W. Lawson, toxicologist. Unfortunately, some pieces of data are missing, most important of which are the cholinesterase data on the chickens and pigeons. I'm afraid these are irretrievably lost in the files of FDA. With the above explanation in mind I will reconstruct the experiments as best my memory and notes permit and present the data obtained.

These studies were conducted in three phases:

(1) a range-finding and screening study with chickens,

(2) a long-term study with chickens, and

(3) a long-term study with pigeons.

I will describe them for you in that order.

Range-Finding and Screening Study

These are the studies which were reported at the 1956 Pharmacology meeting and published only as an abstract in Federation Proceedings.

In all cases the compounds were dissolved in corn (maize) oil, mixed into commercial laying mash, and made available to the birds ad libitum. All chickens were laying hens approximately one year of age. My memory tells me they were Rhode Island Red strain. Each insecticide was incorporated in the diet at a relatively low concentration and increased after several weeks if clinical signs of neurotoxicity or severe toxicity due to cholinesterase inhibition did not appear. In either case the clinical signs were allowed to progress until the animals were sacrificed in extermis. The compounds included in the screening study were tri-orthocresyl phosphate (TOCP) as a positive control, EPN, parathion, malathion, and demeton.

Table 1 gives an outline of the screening study showing the number of chickens, highest dietary level achieved and the duration in weeks that the chickens were fed the respective compounds. Because most of the chickens eventually became too weak to eat and became severely emaciated before sacrifice, a separate group of control chickens were placed on a restricted diet to duplicate any pathologic changes that might be due to malnutrition. Table 2 shows the weight at sacrifice for the various groups in relation to controls. It can easily be recognized that all of these levels are high levels which would be expected to cause marked cholinesterase inhibition. My incomplete records show that the initial feeding levels of parathion and demeton was 100 ppm and that the level was doubled every 2 or 3 weeks. Memory suggests that the initial level for EPN was 300 ppm, 750 ppm for malathion because of their lower acute toxicity. I believe TOCP was started at the 2,500 ppm level.

It should be noted from Table 1 that the TOCP chickens reached an extremis situation sooner than the others, and the EPN chickens at an intermediate time to the others. It is also noteworthy that despite the known lower toxicity of EPN to other species, chickens could not tolerate as high a dietary level as the other pesticides.

The reason for this is shown in Table 3. The TOCP chickens developed an inability to stand after 2 weeks and consequently were unable to reach their feed and rapidly lost weight. The EPN chickens did not show the same rapid onset of leg paralysis, but did develop clinical signs of muscle weakness and inability to stand before other toxic symptoms predominated. The malathion chickens gave a slight suggestion of specific leg weakness after many weeks of feeding. Parathion and demeton chickens showed no symptoms other than those expected from a cholinesterase inhibitor.

At autopsy, the only gross pathologic change which was related to treatement was emaciation which was graded from marked to extreme in all birds except the TOCP and negative controls groups. Microscopic changes which were related to treatment were observed only in voluntary muscle, sciatic nerve and spinal cord. In addition to the usual formalin fixative with hematoxylin-eosin stain for most organs including part of the sciatic nerve and upper spinal cord, the rest of the sciatic nerve and lower spinal cord was fixed with Orth solution and stained with osmic acid. No treatment-related microscopic changes were detected except in the TOCP and EPN groups. With both groups voluntary muscle tissue showed very slight to slight shrinkage of muscle fibers and multiplication of nuclei. The changes observed in nervous tissue are shown in Table 4. In the case of TOCP, the sciatic nerve of all chickens showed fragmentation and lysis of axons, and swelling of nerve fibers. The spinal cord of 2 chickens showed similar changes with oxyphilic irregular masses, probably remnants of degenerated axons. In the case of EPN, the sciatic nerves showed the same changes as TOCP but moderate myelin degeneration was present in one chicken. However, unlike TOCP the spinal cords of the EPN chickens were normal.

At the completion of this study, we were convinced that we could clinically diagnose leg weakness and paralysis caused by nerve damage as distinguished from generalized weakness due to the pharmacological action of anticholinesterase. Although we had fed excessively high levels, we had demonstrated that neurotoxic effects could be produced by subacute administration of TOCP and EPN. Some leg muscle weakness had been observed with malathion, but no pathologic changes were found in the nervous tissues.

The only compounds which demonstrated any tendency to neurotoxicity subacutely were those compounds which had been demonstrated previously to respond to an acute dosage.

Long-Term Study with Chickens

Based on the clinical and histopathological observations of the range-finding study, it was decided to conduct a long-term feeding study with chickens at a series of lower feeding levels with TOCP and EPN. These experiments were initiated in March and April 1956 and were terminated in November and December after 7 months. Because we had recently discovered the phenomenon of potentiation of the cholinergic properties between EPN and malathion, we wondered whether or not potentiation might extend to neurotoxicity. Therefore, later in August 1956, we added groups of chickens on dietary combinations of EPN/TOCP and EPN/malathion. These were also terminated in November and December after approximately 4 months.

In this experiment, female chickens approximately 2 years of age were used. They weighed an average of 2.7 kg and experimental groups consisted of 4 birds each. The experimental compounds were incorporated into the diet in corn oil solutions and fresh diets were made available three times a week ad libitum. All chickens were autopsied but unfortunately no microscopic pathology reports can be located. I do not consider this a serious loss because in the range-finding study, we had been able to correlate clinical symptoms with histologic changes in nervous tissue. In fact, histologic changes were not detected in any animal which had not exhibited clinical signs of neurotoxicity.

Table 5 lists the feeding levels administered to the chicken for TOCP, EPN and the combinations, show the mortality observed with each group and the time of death. The highest level of TOCP, 800 ppm, produced rapid onset of neurotoxicity and death in 4 to 7 weeks. One of the chickens on the highest level of EPN, 400 ppm, and one on the combination of 50 ppm of EPN and 50 ppm of TOCP also developed early symptoms and died in 4 to 6 weeks. One 400 ppm EPN chicken also died early due to an accident. All of these birds lost weight rapidly and died in a severely emaciated condition. All other chickens which developed symptoms and died, exhibited a more delayed pattern, with death occurring generally after 3 to 6 months on diet. In some of these later deaths, the first symptoms of neurotoxicity did not appear until 5 months of feeding, both with TOCP and EPN. No chickens developed initial symptoms later than 5 months.

Table 6 lists the progression of symptoms as they developed from "slight" to "moderate" and to "severe". This is the basis for reporting the severity of symptoms that are reported in Table 8. The most characteristic early symptom we observed was curling back of the toes. This would interfere with the chicken's ability to stand erect and to walk normally. Soon after this the chicken would start to rest on its tarsus or hocks, and food intake would decrease presumably because walking was difficult. Frequently, diarrhea would be present at this moderate stage. As the symptoms progressed, the chicken would no longer be able to maintain this upright position on its hocks and would roll over on its side. Even with help it would be unable to stand and food intake would be reduced to almost zero. The bird would develop severe emaciation and death was probably due to starvation.

Table 7 provides a tabulation of the average weight gain or loss for each group and the approximate food intake. These are terminal weights and reflect the severe emaciation which developed in the two highest groups of TOCP and EPN. In these cases body weight loss was between 30 and 54 percent. Food intake was appreciably low throughout the experiment for these groups.

Table 8 shows the severity of symptoms which developed for each bird in each group. Obviously, the response is dose related and at the lowest levels for TOCP and EPN, the symptoms are slight. With one exception, only occasional slight symptoms were noted with all of the combination diets. Unfortunately, the combination diets were administered for only 4 months, and delayed symptoms were observed as late as 5 months with the individual compounds.

On the basis of 7 months feeding to chickens, these experiments confirm a minimum neurotoxic effect at dietary levels of 100 ppm TOCP or 50 ppm EPN. On the basis of 4 months feeding, a combination diet of 25 ppm of TOCP plus 25 ppm EPN as well as 20 ppm EPN plus 500 ppm malathion was with no neurotoxic effect. No evidence of potentiation of neurotoxic effects was uncovered.

Pigeon Feeding Study

The purpose of this study was to develop sufficient information to determine whether or not pigeons could be substituted for chickens as a suitable animal model for neurotoxicity. The potential advantage from their smaller size would be the opportunity to use large number of animals.

Accordingly, three groups of ten birds each were fed diets containing TOCP at 750, 225 and 75 ppm. Five groups of ten birds each were fed EPN at dietary levels of 200, 100, 30, 10 and 3 ppm in laying mash. The pigeons were provided the diets for 5 months unless they died or were sacrificed sooner. Microscopic pathology was performed on representative pigeons exhibiting leg paralysis. Table 9 summarizes the results by presenting the incidence of symptoms and time of onset. Most of the pigeons fed 750 ppm TOCP showed no symptoms until about six weeks when leg paralysis developed. The other feeding levels produced no symptoms in the 5 months of feeding.

The pigeons fed 200 ppm EPN developed leg paralysis more rapidly and all died or were sacrificed within 4 weeks. An average of 20% loss in body weight was seen in those birds which did not die promptly. At 100 ppm two pigeons were apparently paralyzed in 10 and 14 days, but the remainder showed no symptoms throughout the study.

Microscopic examination failed to confirm any significant changes in sciatic nerve or spinal cord either with hematoxylin/eosin or osmic acid stained sections.

In general, the symptoms observed with pigeons did not appear to progress through stages as with the chickens. The pigeons were either lying down or not, apparently with leg paralysis. In view of the absence of confirmatory lesion by microscopic pathology, the symptoms observed may have been due to cholinergic properties, rather than specific neurotoxicity. We concluded that the pigeon was less sensitive than the chicken to neurotoxic effects of organic phosphates and consequently a less suitable species than the chicken.

Conclusions

It was concluded that:

1. Acute studies using near lethal doses was an adequate qualitative screen for identifying organic phosphates capable of producing neurotoxicity.

2. Neurotoxicity was a dose related toxic effect which was cumulative in chickens up to 5 months.

3. The minimum neurotoxic dose of EPN was higher than the minimum dose producing cholinesterase inhibition. Tolerances for EPN residues on food had been established on the basis of cholinesterase inhibition and thus had been based on the most sensitive index of toxicity.

4. There was no evidence of potentiation of neurotoxic effects from simultaneous exposure to combinations of organic phosphates.

5. Pigeons were less sensitive than chickens to the neurotoxic action of organic phosphates and a less suitable animal model for man.

On the basis of these studies, FDA adopted a policy that any organic phosphate which produced delayed neurotoxic effect in the acute screen should be studied in a long-term (6 month) chicken study to define the no toxic effect level. Such studies would not be required on those which failed to produce neurotoxic effects in the acute screen.

Table 1. Outline of Screening Study

Compound	No. of Chickens	Highest Level -ppm	Duration Weeks
Control	9	0	7-40
Control R.D.*	3	0	7-10
TOCP	2	2,500	3-4
EPN	4	600	5-14
Parathion	4	1,600	13-17
Malathion	4	10,000	15-17
Demeton	4	1,600	14-20

*Restricted Diet

Table 2. Sacrifice Weight of Chickens

Control	2.7 kg	Parathion	1.1 kg
Control R.D.	1.1 kg	Malathion	1.2 kg
TOCP	1.9 kg	Demeton	1.1 kg
EPN	1.0 kg		

Table 3. Clinical Effects Noted

Control: no clinical abnormality

Control R.D.: progressive loss in weight, generalized muscle weakness, emaciation, sacrificed in extremis

TOCP: inability to stand after 14-15 days, although able to move legs; muscle response to stimuli decreased thereafter, prostrate with slow, gasping respiration at sacrifice

EPN: progressive muscular weakness and ataxia, some tremors and inability to stand; emaciation and little muscular response to stimuli at sacrifice

Parathion: progressive muscular weakness, loss of balance, emaciation, sacrificed in extremis

Malathion: progressive muscular weakness, sudden inability to stand, emaciation, sacrificed in extremis

Demeton: delayed muscular weakness, emaciation, sacrificed in extremis

Table 4. Pathologic Observations - Sciatic Nerve and Spinal Cord

Control: normal

Control R.D.: normal

TOCP: sciatic nerve of all showed fragmentation and lysis of axons, swelling of nerve fibers (trace to marked)

spinal cord of 2 chickens showed swelling, fragmentation of axons, oxyphilic, hyaline-like spheres and irregularily shaped masses principally near anterior median fissure.

EPN: sciatic nerve of all showed fragmentation and lysis of axons, swelling of nerve fibers (trace to marked), myelin degeneration in one chicken

spinal cord normal

Parathion: normal

Malathion: normal

Demeton: normal

Table 5. Mortality - Long-Term Chicken Study

Compound	Feeding Level (ppm)	Mortality Ratio	Time of Death (weeks)
TOCP	800	4/4	4,4,5,7
	400	3/5	15,16,19
	200	0/4	-
	100	0/4	-
EPN	400	4/4	4,5,13,14
	200	3/4	9,12,17
	100	2/4	20,20
	50	1/4	24
TOCP/EPN	50/100	0/4	-
	50/50	1/4	6
	25/25	0/4	-
	10/10	0/3	-
EPN/MAL	40/1000	0/4	-
	20/500	0/4	-
CONTROL	-	1/9	8

Table 6. Description of Progressing Symptoms of Delayed Neurotoxicity

Slight	Uncoordinated gait
	Failure to stand on toes
	Toes curled
Moderate	Rests on hocks (tarsus)
	Diarrhea
	Reduced food consumption
Severe	Rests on side
	Uncoordinated movements
	Emaciation

Table 7. Long-Term Chicken Study - Body Weight and Food Intake

Compound	Feeding Level (ppm)	Weight Gain/Loss (%)	Approximate Food Intake (g/day)
TOCP	800	-35	60
	400	-30	90
	200	-17	160
	100	+ 7	170
EPN	400	-54	60
	200	-42	90
	100	-11	130
	50	- 4	140
TOCP/EPN	50/100	- 8	-
	50/50	-10	-
	25/25	+ 3	-
	10/10	+18	-
EPN/MAL	40/1000	-19	-
	20/500	0	-
CONTROL		0	160
		+11	150

Table 8. Clinical Symptoms Observed in Long-Term Chicken Study

Compound	Feeding Level (ppm)	Symptoms	
TOCP	800	severe	4/4
	400	severe	5/5
	200	severe	2/4
		moderate	2/4
	100	slight	1/4
		none	3/4
EPN	400	severe	3/4
		accidental death	1/4
	200	severe	3/4
		slight but severe emac.	1/4
	100	severe	1/4
		moderate	1/4
		slight	1/4
		none	1/4
	50	moderate	1/4
		slight	2/4
		none	1/4
TOCP/EPN	100/50	slight	3/4
		none	1/4
	50/50	severe	1/4
		slight	1/4
		none	1/4
	25/25	none	0/4
	10/10	none	0/3
EPN/MAL	40/1000	slight	1/4
		none	3/4
	20/500	none	0/4

Table 9. Pigeon Feeding Study - Five Months Mortality and Symptoms

Compound	Feeding Level (ppm)	Symptoms	Time of Symptoms (weeks)
TOCP	750	10/10	6
	225	0/10	-
	75	0/10	-
EPN	200	10/10	2-4
	100	2/10	2
	30	0/10	-
	10	0/10	-
	3	0/10	-
Control	-	0/16	-

DR. J. SANBORN: Could not some of the weight loss that you observed have been due to the palability of the food? I venture to say that with a 1% incorporation of some of these things in the diet, I don't think too many of us would find that very palatable.

DR. J. P. FRAWLEY: These levels were not levels that had not been accepted by other laboratory species. In all cases -- rats and dogs, at least, in our own laboratory, had ingested levels approaching those. I can't deny that it's possible that some of the emaciation might have been due to food refusal. But the chickens, in nearly all cases where the greatest emaciation occurred, were laying around not showing much interest in doing anything else. I suspect they were just sick.

DR. F. H. DUFFY: Has anyone tried electrophysiological studies on the chickens? It seems to me that if they can be used, they might save you a lot of time and trouble in becoming chicken clinicians. It doesn't cost very much to get a device that will shock a chicken's foot and measure resultant nerve potentials from the neck. One could determine a combined nerve and spinal cord conduction time. This correlates, usually, very well with the neuropathology, and would seem to be more sensitive than watching a chicken stagger. You could probably pick up conduction velocity abnormalities prior to the onset of any clinically evident material. It's not a very expensive or difficult thing to do. I just wondered if anyone has tried it in the chicken and correlated it with the clinical results?

DR. J. P. FRAWLEY: Not to my knowledge. But I think it's a fine suggestion.

DR. S. H. FRAZIER: You said that the dose for neurotoxicity is cumulative. Are you saying that you can give a certain level and on continuing to give that level the material would accumulate in terms of not metabolizing out and you would get neurotoxicity? Or are you using the term cumulative in another context?

DR. J. P. FRAWLEY: I'm not talking about biochemical accumulation or of a material constantly building up in the body with absolutely no elimination. Cumulative toxicity doesn't necessarily mean one plus one plus one equals three. You get some increased pharmacological or toxicological response from subsequent doses. But the cumulation levels off, as we've seen in these animals, and it does so in most cases of cumulative toxicity. You reach some equilibrium point where metabolism is compensating for the intake, and you have no further, either biochemical accumulation or lesion taking place in the body.

DR. S. H. FRAZIER: In consideration of the safety of residues in foods, from your results is the neurotoxicity parameter one of a more primary concern than cholinesterase inhibition, as far as ADI is concerned?

DR. J. P. FRAWLEY: I don't know of any case where neurotoxicity has occurred at a lower level than causes that causing cholinesterase inhibition. I was maintaining that the ADI's that the WHO has set, the tolerances that FDA and EPA has set, have usually been based on the most sensitive index of toxicity, which is cholinesterase inhibition, not neurotoxicity. So that if you're protecting against cholinesterase effect, it seems to me that you're not allowing any neurotoxicity to take place.

DR. OLSON: Many of the symptoms that you described in your birds strongly resemble a great many of the symptoms of nutritional deficiency seen in poultry in the early nutrition research days from the late '30's. I think a lot of your emaciation would induce this, and perhaps to such an extent that your pathology and other observations might have been complicated by nutritional deficiencies. This might not be seen so much with a modern day type of diet. I'd appreciate your comments on that.

DR. J. P. FRAWLEY: Well, certainly at the high levels these animals did become emaciated. At the intermediate levels they were eating well. I don't think that they were suffering from severe nutritional deficiencies. Certainly I agree with you that at high levels those birds were down. And perhaps if someone wanted to repeat this kind of a study today, it might be better to intubate the animal to administer the material.

DR. M. K. JOHNSON: I was encouraged to find you bringing this information out. It agrees extremely well, I think, with that which Professor Cavanagh has quoted as unpublished work, which I have then pirated and quoted in the review in Critical Reviews in Toxicology. With 800 parts per million they got gross ataxia in 16 days. And the birds had then only had about 0.45 mg/kg, which means something about 0.6 gm/kg. So there was very great cumulative effect. That's not much more than the single acute dose.

DR. J. P. FRAWLEY: This is TOCP?

DR. M. K. JOHNSON: Yes! And then, when they got down to 100 ppm, in agreement with you, they couldn't find an effect in 150 days. I calculated the dose as about 0.7 ml/kg (equal to 0.84 mg/kg) at that stage, for which they showed no effect. I think it's interesting, as you've pointed out that there is a quite rapid cutoff as one goes down, to a point where there is no effect.

Now, to make two points about that. First, that if you spread the doses out instead of giving daily doses then you get to the stage where there appears to be no cumulative effect at all of substantial doses which are more than three weeks apart. Dr. Barnes found this because in routine testing he usually waited three weeks to see if anything would happen, and then gave another dose. Whenever doses were more than four weeks apart we never saw anything happen in response to the second dose which hadn't happened on the first dose.

Secondly, it seems to me it would be nice, as you pointed out, to know what is happening on the enzymic level in these chronic feeding cases. You could put the question in this form. Does chronic feeding, say, of 200 ppm or something like that level, keep the enzyme at 50% inhibited for a long period, or does it manage to work its way up, slowly increasing inhibition until you get up to 80 to 90 percent inhibition? And I think it would be nice if anyone were willing to repeat such a chronic study, if they could at intervals monitor the enzyme levels.

I think perhaps on a thing like that it might be necessary to go directly and monitor, say, the spinal cord rather than the brain, just in case there were a small variation in the actual percentage inhibition. And I have one question to raise. Your data and Dr. Barnes's data agree very well. Dr. Kimmerle has recently noted histological damage obtained on chronic feeding at only 10 ppm. It does bring to my mind this question about what exactly do we mean when we say TOCP. I guess neither you nor Dr. Barnes would be able to say precisely what was the composition. I wonder if the material which Dr. Kimmerle has used has been chemically defined very precisely. And whether he could tell us if those effects -- or how they related to the single dose activity. In other words, was it in fact an unusually active preparation in both respects.

DR. J. P. FRAWLEY: I certainly don't have any idea on the purity of the TOCP we used in those days, and I doubt if the sample could ever be retrieved. But I would like to reflect for a minute on one of the things that John Casida brought up earlier. That these compounds inhibit a whole series of esterases. I think to properly investigate the toxicology of these compounds, you should be looking at the effect on the whole series of esterases that we can identify. That should include not only the pseudo and true cholinesterases, but the aliphatic esterases, to see whether or not there's a propensity for interfering with metabolism with some other compounds as well as the neurotoxic esterase system.

I think it's a whole series of esterases. When we know how these compounds affect them, we should look at them, before deciding on a safe level.

Does Dr. Kimmerle want to comment on the TOCP purity situation in his studies?

DR. G. KIMMERLE: I don't know the actual purity. It was a technical mixture.

DR. M. D. ABOU-DONIA: I would like to comment on the effect of leptophos, since EPN is very similar to leptophos in structure, both being phenyl phosphonothioate compounds. From our experiments with hens, we found that in both single dose administration and in long-term chronic oral administration of leptophos, we obtained a dose response relationship. However, there were distinct differences in both treatments. In the case of a single oral dose, the no-effect level was 140-160 mg/kg body weight. An acute oral dose of 180 mg/kg resulted in neurotoxicity. Those chickens that developed ataxia, developed a paralysis and ultimately died. Clinical neurological signs of poisoning coincided with positive histological findings on examination of nerves.

The basic difference between the single dose and the long-term chronic administration is that in the case of a single dose that there was always an all or none effect. Either there is no observable effect or if there is an effect it will always proceed along the line of ataxia, paralysis and death. In all cases, the clinical conditions correlated with histopathological changes. In the case of the chronic feeding, there were in some instances at the lower level, some recovery from paralysis. In the case of a single dose, ataxia always led to paralysis and to death. In those levels of chronic dosing where there was a recovery from paralysis following ataxia, no histological changes were evident. The effect could have been solely at the molecular or biochemical level which had not progressed to be observed on histopathology.

We did examine the effect on the enzymes and found that on the pseudo cholinesterase enzyme was definitely inhibited. Even though after we stopped the dosing of the chickens, the inhibition still continued.

DR. J. P. FRAWLEY: Well, I don't see that anything you've described is particularly unusual in toxicology. I mean, in an acute dose you're overwhelming the system. When you overwhelm a system it's hard to pick up individual variations in animals. In your sub-acute study you're titrating from the bottom and you're going to find the effects of individual members of a strain or a species. So, you'll have more variation in a sub-acute feeding of that nature than when you just overload the whole system as with 180 milligrams per kilogram.

DR. M. B. ABOU-DONIA: What was interesting was the fact that the effect was not just cumulative, but more than cumulative, much more of an effect.

DR. J. P. FRAWLEY: Well, again, that's not unusual to see in sub-acute and chronic studies.

DR. A. W. BRADLEY: I notice you found de-myelination in one of your birds. I would suggest that this was probably a false positive, actually. When you look for demyelination lesions, fixation is important. I purposely didn't mention this in my discussion previously because these are specialized techniques that are not really suitable for routine examination. You need proper perfusion, and this means putting between six to either litres of perfusion fluid through the bird, which takes from two to three hours to do. Hence, to look for demyelination in a preparation that's not been properly fixed, will often lead to false positives.

The common staining technique that has been used is the Marchi method. When using a technique like the Marchi method, if you use paraffin sections you will not show demyelination in carefully prepared control sections.

Another comment on demyelination, some people are still referring to delayed neurotoxicity as a demyelinating disease. It isn't! I think it's twenty years since Cavanaugh showed it was an axonal degeneration, and yet, people are still looking for demyelination which I don't think is really applicable. It's far better to look for axonal degeneration which is easiest to spot with some fairly simple techniques.

DR. D. V. ROBERTS: Could I just take advantage of your open invitation for comments to say that what we are doing with the ENMG is basically, to provide another bit of information concerning a physiological parameter of nerve and muscle function. We believe the real value of the ENMG at the moment is a practical one, in the sense that we can measure this parameter and then assess whether or not changes have occurred. Then we can go quickly back to the plant and say to the people working there, "We believe that your exposure is more than it should be. Would you like to check the working conditions?" We find that when we do this, time and time again, they are able to come up with an explanation of the overexposure and to correct it. Then if we examine the workers over the next few weeks we find that they come back into the normal range. And of course, there is no hazard to health in all this.

It is right at the subclinical level. From our experience we feel we are catching the effects long before they ever get to the state of irreversible changes, and to us, this is the main point of the exercise. The other thing that I would like to say is that the effects we see are not specific for cholinergic action. Nor are they specific for delayed neurotoxic effects. They reflect functional changes which may be produced by many compounds. The method has a wide application and should not be seen to relate more to delayed neurotoxic effects than to other factors influencing nerve and muscle function.

DR. E. REINER: I am myself not involved in the EMG studies on workers exposed to pesticides. But, as this was one of the topics discussed here, I may perhaps say a few words about Dr. Anica Jusic's work. She is a colleague in our institute in Zagreb, who is trying to establish if there are some alterations in the EMGs of workers exposed to pesticides. What she has done is to use needle electrode techniques to measure the EMG. As far as I know, she has found no alterations in the workers who were exposed for a long time to pesticides, particularly OP compounds and carbamates, when she compared the EMGs of those exposed people with the control group.

At the same time, she did her examinations, the cholinesterase levels of these people were measured. These levels were decreased only by about ten or fifteen percent of their own controlled pre-exposure values. So one can argue, have these people absorbed any compound to any appreciable extent? A decrease of something like 10 or 15 percent can also occur normally in people. It doesn't necessarily mean an inhibition due to absorption of an anti-cholinesterase compound.

But, if one does assume that they have absorbed the compound, then Dr. Jusic's conclusion is that one cannot find EMG changes in those people who worked for a considerable time with anti-cholinesterase pesticides.

DR. D. V. ROBERTS: This is an interesting difference between two techniques, one using surface electrodes, in which we are essentially looking at a population of nerve and muscle fibres, and the intramuscular technique used by Dr. Jusic in which observation is restricted to a very small number of muscle fibres. And it may well be that, in fact, the ENMG changes that we see, which are perhaps due to changes in nerve function, would not be reflected in the parameters of EMG by the conventional needle technique. There is one additional point and that is that Dr. Erik Stolberg of Uppsala has a nice technique which is a very sensitive one for detecting abnormalities in function, particularly of the nerve terminals. He has found evidence in a small number of workers who have been engaged in spraying a variety of compounds of the organophosphorus type, of a denervation type of lesion. I mention this because we need to bring all the information together. If we use different techniques we may, in fact, get different bits of information but these will, in the end, make up a complete picture. I think that Dr. Jusic's work is useful but it has to be taken in the context of intramuscular recording, which of course means that needles have to be put into muscles and the chances of doing a sequence of recordings over many weeks is not quite as good as it is with the surface recording which we use.

DR. E. REINER: It is difficult for me to discuss Dr. Jusic's results, but if I recall correctly, Dr. Jusic claims that she can't get very reproducible results if she uses surface electrodes. If she examines and re-examines a given person several times, it is a different group of fibers in a different position which she does record. And she claims that even in a control group where there's no record of any exposure to pesticides, there's a tremendous variability in the EMGs which she does record when using surface electrodes. Therefore, the variability is such that in the exposed group, any alterations will somehow disappear due to the alterations of the method. This is the reason why she used needle electrodes, where she says she can get much more reproducible results and be certain that it is just the potential of one particular fiber which she can identify by putting her needle into it.

That's her interpretation and I can't discuss whether it's correct or not.

DR. D. V. ROBERTS: Yes. Reproducibility is important. I happen to know that Dr. Jusic does not, in fact, use the same muscle that we do, which we chose because with it, we can get more reproducible results. In fact, if you take a number of non-exposed individuals and do their EMG's once a day for three weeks, the average coefficient of variation of values you obtain is seven percent, which is not too bad for a biological function.

ORGANOPHOSPHATE EXPOSURE FROM AGRICULTURAL USAGE

John E. Swift, Ph.D.

This discussion is supposed to be concerned only with the exposure of humans to organophosphate compounds and the results of these exposures. However, it is not possible for me to confine this subject to just organophosphorous compounds and cover the problem of pesticide exposure and related illnesses. The type and number of injuries is most often determined by the type of work a person is doing at the time of exposure.

These remarks will be confined to the situation in California as that is where these statistics are from and also I read in a recent EPA summary that Region IX has reported the greatest number of pesticide related illnesses. The reasons for this are hard to define; is it because we are more careless than others, use more pesticides, do a better job of reporting illnesses or do we have special environmental or climatic conditions that influence some episodes of pesticide poisoning? I am inclined to believe that all of these factors may contribute to this problem.

The reporting system as directed by the California Workman's Compensation Law states that each physician who attends an employee who is ill or injured as a result of a work practice must file a report on that injury, this is with the California Department of Industrial Relations, this is called the "Doctor's First Report of Work Injury". This agency then routes these reports to the California Department of Health who compile these statistics, assign classifications of work injury with the Department of Food and Agriculture investigate as many cases as possible they then publish an annual report. Under this system about 85 percent of the injuries in the work force are covered, but it does not include the farmer, unpaid family labor, self-employed pest control operators, those not under Workman's Compensation which may exclude some of the non-U.S. citizen field workers.

Some definitions are in order to clarify some of the points that will be covered during this discussion: for this reporting a systemic illness refers to a generalized illness that involves more tissues than eyes or skin and usually two or more body systems; however, this classification can involve only the respiratory system or a generalized allergic reaction. Skin condition or dermatitis refers to a reaction of the skin, excluding abrasions and thermal burns. Eye condition refers to any condition of the eye caused by a chemical substance. Eye and skin condition refers to cases that have both of these.

The occupation or job requirements appear to be almost as critical in injury cases as the chemicals involved. The classification of agricultural workers in regards to their potential exposure and injury is as follows: ground applicator; mixers and/or loaders; gardeners; field worker exposed to pesticide residues; nursery or greenhouse workers; soil fumigators in agriculture; tractor drivers or irrigators; cleaners and/or mechanics of application equipment; worker exposed to drift from the application site; aerial applicators (pilots), flaggers for aerial application and others.

The workers most frequently reported as being ill from pesticide poisoning are the ground applicators and the mixers and loaders. In some cases one person could be doing all of these jobs; however, for reporting purposes such injuries are divided into these two job specialties.

In order to bring out the type and incidence of poisonings as they relate to job specialties and chemicals involved, I will briefly go through some of the accidents in some of these categories in 1974. There were a total of 1,157 reported cases to the Department of Health in 1974 which was a reduction of 117 cases from 1973; however, I hardly believe this is significant. These incidences are not investigated in detail except for those workers exposed to pesticide residues at harvest or some other job involving intimate contact with the treated foliage.

Ground applicators have had more illnesses than any other agricultural occupation. The following will show some of these occurrences.

(1) GROUND APPLICATORS - 229 CASES - 1974

SYSTEMIC CASES - 92

Organophosphates (Parathion or Phosdrin-37)	55	Roundup	1	Dowpon	1
		Ansar	1	2,4-D	1
Carbamates	8	Kelthane	1	Ro-neet	1
Dinitro	3	Princep + Simazine	1	Mixtures	5
Paraquat	2	Sulfur	1	Unknown	11

Organophosphates most commonly involved in more serious systemic illnesses.

Spilling concentrate on skin while mixing is a major problem.

Other factors:

1. Drift onto operator while spraying
2. Lack of safety equipment
3. Excessive heat which causes the worker not to use the safety equipment
4. Poor training
5. Long working hours
6. Intimate contact with the spray materials and concentrates.

(2) GROUND APPLICATOR - 1974

DERMATITIS - 66 CASES

Weed Oil	13	Paraquat	3	Omite + Guthion	1
Omite	4	Eptam	1	Malathion+Acaraben	1
Comite	3	Alfa-tox	1	Dormant Oil + Bluestone	1
Treflan	1	DD	1		
Dinitro	1	Zinc	1	Paraquat + Keramax	1
Ansar	1	Lime	1	Omite + ZNP	1
Sulfur	3	Dibrom + Omite	1	Mixture	3
Trysben	1	Omite + Sulfur	1	Unknown	20

Omite and Comite involved in a large percentage of cases in 1974 and even more in 1975.

Drift of spray on applicator caused 10 cases.

Lack of safety equipment caused 7 cases.

Material splashed on applicators.

(3)

GROUND APPLICATORS - 1974

EYE INJURIES - 67 CASES

Sulfur	10	Dow General Weed Killer	1	Difolatan	1
Weed Oil	8			Parathion	2
Paraquat	2	Tillam	1	Diazinon	1
Contact	2	Balan	1	Omite + Benlate	1
Dinitro	3	Omite	1	Omite + Dibrom	1
Ansar	1	Toxaphene	1	Methyl bromide	1
Bravo	2	Eptam	1	Knox-out	1
2,4-D	1	Plictran	1	Mixture	3
				Unknown	20

Drift in eyes of applicator a major problem - refused goggles.

Repairing hose and adjustments while equipment in operation.

Operator rubbing eyes without washing first.

Splashing material into eyes.

(4)

GROUND APPLICATORS - 1974

EYE AND SKIN INJURY - 13 CASES

Omite	3	Enide	1
Comite	1	Omite + Sulfur	1
Weed Oil	1	Unknown	6

Common cause of injury drift of pesticide from point of application back to and on applicator.

It is obvious that the most serious illnesses are caused by the organophosphorous compounds, but carelessness with other materials causes some illnesses. It should be noted that parathion and phosdrin are the worst offenders of the organophosphorous materials. Lack of protective equipment and clothing is the basis for a number of injuries and the excessive heat certainly contributes to this. The other types of illnesses seem to be caused by almost anything that is used, and the refusal to wear long-sleeved shirts, goggles and a hat apparently is a contributing factor.

The mixer/loader group has the next highest number of illness and apparently for the same reasons as the applicator except since they do not do actual spraying they are not bothered by the spray drifting on them. Their intimate contact with the concentrates is a major factor in these illnesses.

(5) MIXER AND/OR LOADER - 142 CASES - 1974

SYSTEMIC ILLNESSES - 75 CASES

Organophosphates (Parathion & Phosdrin-39)	46	Lead Arsenate	1	Sulfur	1
		Dinitro	1	Herbicide	1
Carbamates	8	DD	1	Mixture	3
Broadside	1	Paraquat	1	Unknown	11

Organophosphates most frequently implicated in severe illnesses.

Most illnesses caused by direct contact with the concentrate.

Lack of use of safety equipment - most refuse goggles and gloves.

Inhaling dust from W. P. concentrates.

Splashing or spilling the concentrate on themselves during the mixing or loading process.

(6) MIXER LOADER- 1974

DERMATITIS - 20 CASES

Telone	2	Ordram	1	Dyrene	1
Omite	2	Weed Oil	1	Carbamate	1
Dibrom	1	Nemagon	1	Paraquat	1
Sulfur	4	Chlordane	1	Unknown	4

Most injuries are a result of spillage on the skin.

(7) MIXER LOADER - 1974

EYE INJURY - 40 CASES

Sulfur	2	Eptam	4	Telone	1
Lannate	1	Tillam	1	Hydrated Lime	1
Omite	3	Thimet	1	Toxaphene	1
Comite	1	Copper monohydrate	1	Difolatan^R + Sevin	1
Weed Oil	3	Urea	1	Eptam + Treflan	1
Ansar	1	Ethyl Dibromide	1	Derinol + Planivin	1
Paraquat	2	Sodium Arsenate	1	Organophosphate	1
Roundup	1	Phosdrin	1	Mixture	1
Dinitro	3	Parathion	1	Unknown	3

Herbicides caused a number of problems.

Fourteen injuries caused by splashing concentrate or dilution onto worker - refusal to wear goggles.

Inhalation W.P. concentrate - Hose malfunction and carelessness.

(8) LOADER/MIXER - 1974

EYE AND SKIN INJURIES - 7 CASES

Weed Oil	1	Sulfur	1
Dinitro	1	Mixture	1
Thiodan	1	Unknown	2

Field workers exposed to pesticide residues may be the most difficult situation to rectify. These people are those who thin, prune and harvest the crops and have substantial contact with treated foliage. They enter a field to do a specific job and frequently they do not know that the plants have been treated with a pesticide. Even if they did, there is not too much they can do to protect themselves except to wear protective clothing, long sleeved shirts, hats and gloves. Occasionally there are a large number of people made ill at one time and we don't have an answer yet except in all recorded cases organophosphorous compounds are involved and usually ethyl parathion is the chemical usually implicated. I will discuss this later.

Excluding systemic injury as a result of organophosphorous compounds, both eye and skin injury frequently involved sulfur.

(9)

FIELD WORKERS EXPOSED TO PESTICIDES RESIDUES - 117 CASES - 1974

SYSTEMIC ILLNESSES - 11 CASES

Guthion + Zolone	2	Parathion + Malathion	2
Sevin	1	Unknown	3
Sulfur	3		

Four cases occurred during the picking of grapes.

Two cases followed a pruning operation of grapes.

Fortunately we had no serious cases of systemic poisonings during 1974.

(10) FIELD WORKERS EXPOSED TO PESTICIDE RESIDUES - 1974

DERMATITIS - 77 CASES

Sulfur	18	Benlate + Dithane	1	Cryolite	1
Benlate	1	Acaraben + Unicide	1	Dyrene	1
Benlate + Cygon	1	Plictran + Imidan	1	Seven + Sulfur	1
Benlate + Omite	1	Difolatan	1	Mixture	3
Benlate + Plictran	1	Dalpon	1	Unknown	44

Pruning and tying grape vines accounted for a number of illnesses.

Several cases of injury occurred in the harvesting of crops: 5 in vineyards; 3 strawberries; 2 lemons, 2 celery; 1 squash; 1 pear, 1 cauliflower.

(11) FIELD WORKERS EXPOSED TO PESTICIDE RESIDUES - 1974

EYE INJURY - 18 CASES

Sulfur	11	Sulfur + Captan	1
Omite + Diazinon	1	Unknown	3
Sulfur + Sevin			

Six injuries occurred during pruning and harvesting grapes and five during celery harvesting.

As noted, sulfur is involved in almost every case.

(12) FIELD WORKERS EXPOSED TO PESTICIDE RESIDUES - 1974

EYE & SKIN INJURY - 11 CASES

Dyrene	4	Sulfur + Captan	1
Sulfur	2	Unknown	4

Most occurred during harvest of fruit crops.

In unknown injuries sulfur could still be the main problem.

Gardeners are members of another occupation with a high incidence of pesticide caused illnesses. These are professional gardeners, not home gardeners. Some professional gardeners in California are quite careless or uninformed; most of them use hand operated equipment for spraying and they usually are short on protective equipment. Fortunately, however, serious poisonings within this group do not frequently occur because they usually do not have access to the highly toxic materials such as parathion, phosdrin, other organophosphates and some carbamates. In California these chemicals are restricted-use chemicals and require a permit for purchase, possession and use (Table I). In 1974, there was no case of systemic poisoning involving organophosphate compounds; however, there was one reported case of dermatitis involving parathion (which is questionable). There were five other cases of dermatitis or eye injury involving other organophosphate compounds which are not on California's restricted-use list -- specifically diazinon, malathion and metasystox. Most of the materials available to professional gardeners are in the moderate to slightly toxic categories. However, it seems obvious that if there is a way to inflict self-injury by using a chemical, man will find it.

(13) GARDENERS - 101 CASES - 1974

SYSTEMIC ILLNESSES - 5 CASES

2,4-D	2	Weed Oil	1
Vapam	1	Mixture	1

Highly toxic organophosphorus compounds are not allowable in most work of this type.

One injury was from a spill, a second from drift of his own spray and the third from not using any safety equipment.

(14) GARDENERS - 1974

DERMATITIS - 40 CASES

Weed Oil	5	Phytar	1	Diazinon + Princep	1
Roundup	1	Parathion	1	Dizinon + Mercuric chloride	1
Dibrom	1	Diquat	1		
Dalapon	2	Paraquat	2	Malathion + Kelthane	1
Chlordane	1	Amizol	1	Fungicide	1
Dinitro	1	Isotox	1	Mixture	4
Caseron	1	Difolatan	1	Unknown	12

Four illnesses from equipment malfunction. Four from spills of concentrate. Three due to lack of safety equipment. Some from drift of application. One from contact of previously sprayed plants.

(15) GARDENERS - 1974

EYE INJURIES - 53 CASES

Weed Oil	15	Copper sulfate	2	Amitrole	1
Paraquat	4	Sulfur	1	Diazinon	2
Dowpon	1	Simazine	1	Carbaryl + chlordane	1
Diquat	1	Amizol	1	Dalapon + Weed Oil	1
2,4-D	1	Aquathol	1	Roundup + Pramitol	1
Princep	1	Krovar	1	Sodium chlorate + Metaborate	1
Metasystox	1	Benlate	1	Pramitol	1
Insect repellent	1	Rololind	1	Mixture	1
Chlorothalonil (Bravo)	1	Vapam	1	Unknown	8

Equipment malfunction caused a number of injuries (over 15). Drift of spray back into the eyes of the applicator accounted for several cases. Goggles were not used in most cases. Splashes or spills on the operator also caused a number of injuries.

(16) GARDENER

EYE & SKIN ILLNESSES - 3 CASES

Daconil	1
Sulfur	1
Unknown	1

The last category I want to discuss is the nursery and greenhouse workers. This group has a relatively high incidence of pesticide induced illnesses.

(17) NURSERY & GREENHOUSE WORKERS - 75 CASES - 1974

SYSTEMIC ILLNESSES - 11 CASES

Temik	3	Dexon	1
Dinitro	1	Malathion	1
Lannate	1	Amitrol + Lannate	1
Metasystox	1	Unknown	1
Paraquat			

Three of these cases can be attributed to lack of any protective equipment. The others are partly attributed to the mixing process and to spills.

(18) NURSERY AND GREENHOUSE WORKERS

DERMATITIS - 47 CASES

Weed Oil	3	Copper sulfate	1	Temik, Captan + Benlate	1
Benlate	3	Chlordane	1		
Zectran	1	Actidione	1	Captan, Metasystox + Dithane	1
Diazinon	2	Dithane	1		
Paraquat	2	Regulain	1	Lannate + Benlate	1
2,4-D	1	Cygon	1	Benlate + Dithane	1
Kelthane	1	Dexon + Benlate	1	Mixture	1
				Unknown	22

Twelve employees became ill after handling treated plants. Several illnesses occurred after the person had been spraying the material. Benlate was frequently implicated.

(19) NURSERY OR GREENHOUSE WORKERS

EYE INJURIES - 16 CASES

Lime	1	Dibrom	1	Cygon	1
Rololind	1	Azodrin	1	Temik	1
Metasystox	1	Weed-all	1	Benlate + Dazatrol	1
Dexon	1	Banrot	1	Benlate + Omite	1
				Unknown	4

Four injuries by contacting treated plants. Four injuries associated with accidental spills.

One eye and skin injury cause not known.

Considering the conditions of work and the types of materials frequently used this group has a relatively good record and they also use restricted materials. Supervision in nurseries and greenhouses is usually better than among other areas and this may account for the relatively good record.

A number of other occupationally exposed workers are listed according to their work speciality and these are:

OTHER PESTICIDE ILLNESSES ACCORDING TO OCCUPATION - 1974

1.	Soil Fumigators in Agriculture	29
2.	Equipment Cleaners and Mechanics	28
3.	Tractor Drivers and Irrigators	23
4.	Workers Exposed to Drift	22
5.	Pilots	17
6.	Flaggers	6

Those doing soil fumigation most frequently report illness from methyl bromide and chloropicrin or other fumigants, no organophosphates were involved.

Tractor drivers and irrigators reported 22 cases of injury and 5 incidences of systemic poisoning. Four out of these 5 cases involved parathion and 1 thimet. There were no other injuries involving OP compounds. The same was true for those who clean and repair equipment. For those who are injured while cleaning equipment, for the most part it is the result of carelessness or the owner or supervisor did not advise them of the hazard involved in cleaning out the residue associated with these chemicals. If the owner of the equipment properly advised them as to the material in the equipment, there should be few accidents.

In the case of the mechanic repairing a piece of equipment the illness is the result of the applicator or equipment owner or not notifying the mechanic of the type of material that was last used in the machine.

In both cleaning and repairing equipment, 9 of the 24 cases were systemic poisonings and all involved organophosphorous compounds with parathion or phosdrin causing all but one incident. In the repair of equipment most cases involved a welding torch or some other heat source which vaporized the material and resulted in a pesticide illness almost immediately. This can be stopped if a little more respect is afforded these chemicals.

Workers exposed to drift are those who are in the area but not doing one of the jobs previously mentioned. In 1974, eight cases were as a result of aerial application and 12 cases were from ground application. Of the 10 systemic illnesses 9 were caused by organophosphorous compounds and one by a carbamate. For other types of illnesses the offending chemicals were the same as previously reported.

Amongst pilots, 13 of the 17 reported illnesses were systemic poisonings and all but 3 of these were caused by organophosphorous compounds involving parathion, phosdrin, systox, phosvel and guthion; two of the other three were carbamates; the third unknown. Most injuries were the result of equipment malfunctions in the airplane or crashes.

One would think that flaggers would be subject to many exposures and illnesses caused by pesticides, but this is not the case. There were only 6 cases of poisoning in 1974 and 3 of these were systemic caused by parathion or a combination of parathion plus something else. Organophosphorous compounds were not involved in the other three cases. Flaggers move so that drift is always away from them, they move out of the path of the plane as soon as the pilot gets on course and as a result is one of the safest jobs in agriculture where chemicals are involved.

The problem associated with most of the pesticide induced illnesses can be avoided. Of course it is recognized certain types of accident such as an airplane or truck crashes, explosion or fires cannot be anticipated but many others can and proper measures taken to reduce such illnesses. However, the problem associated with worker illnesses due to exposure to pesticide residues on various crops is one that will require considerably more research. Such cases have been recorded in California since 1949 and while re-entry intervals imposed in 1971 (Table II) have reduced the number of such poisonings. They still are occurring only. Only now are the mechanisms by which such poisonings occur, the environmental factors involved and the extent of the problem are being thoroughly studied.

Of the cases recorded since 1949 there have been a total of 26 incidences occurring in 13 different years (Table III). That is to say some years there were no cases and others 3, 4, or 5 incidences per year. The crops involved have been pears, citrus, grapes, peaches, olive, prune and lettuce. Of significance in this report is that out of 26 incidences, 17 involved parathion, 4 parathion plus some other organophosphorous compound, 4 other organophosphorous compounds and 1 unknown. To further complicate the picture, 18 of the incidences involved citrus and 8 the rest of the crops mentioned. A clue to this situation might be the distribution of these incidences in California. The first occurrence was in the Sacramento Valley, then two cases in the Riverside area and all the rest in the San Joaquin Valley.

The one factor in common here is the weather or climatic condition except for an occasional situation, occur during extremely dry conditions from June to September, the humidity is low and usually a large build up of dust on the foliage of the crop. Dr. Spear and others have found that the weathered residue of parathion decay into paraoxon and is associated with the dust on the leaves which termed a dislodgable residues, and this gets onto the upper body of the worker as he harvests the fruit, prunes or some similar job. Paraoxon is also formed in the soil and it appears that a possible contamination of the whole body may occur. It has been determined that poisoning is a result of paraoxon being absorbed through the skin in sufficient quantitites to cause intoxication. Spear et al., so far have concluded that paraoxon is the principal toxic constituent of the weathered residue. The absorbed dose is almost entirely dermal. In citrus crops the fallout of foliage residues is probably of more importance than direct foliar contact.

As a result of studies like this by Dr. Spear and others we may eventually get to the cause and solution of this problem.

As a further study regarding exposure of field workers to organophosphorous residues -- Peoples, Knaak & Maddy studied in 1975 -- collected blood samples of 1,166 persons during the growing season of specific crops in the San Joaquin Valley. These included male and female field workers and non-field workers, but all from the same farming community. There were 416 male and females who acted as controls. This monitoring was done at the height of the harvest season for lettuce, grapes, peaches, and citrus. The farm workers were selected by physicians at Union farm worker clinics. Or those who volunteered to a sign posted in the clinic printed in both English and Spanish saying "Obtain a free test today for pesticide exposure". This means of selecting subjects should insure a relatively representative sample of the farm worker population. Analysis of these results are still under way but so far there is no difference in cholinesterase value between the field workers and the controls. The largest number of persons involved occurred in September at the end of the grape harvest and these did not show any difference from the controls.

Many things are going on in California and the United States in general to reduce the problem of worker injury to pesticide exposure. Some of the significant things are the research by people like Spear, Maddy and Kahn. Injury and accident investigation, re-entry intervals that are imposed after the use of certain chemicals on certain crops, employers' responsibility for training his employees, emergency medical care and medical supervision for those working with certain organophosphorous or carbamate compounds, it is now illegal for a pilot to assist in loading operations, the future requirement of a closed mixing system, shielding of flexible hoses if they go through any vehicle used for ground or aerial application, and monitoring the farm worker population, etc.

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STATE OF CALIFORNIA

DEPARTMENT OF FOOD AND AGRICULTURE

TABLE I

RESTRICTED MATERIALS

Permit required for possession and use

- (a) Certain pesticides containing arsenic
  - (1) Sodium arsenite**
  - (2) Other pesticides containing inorganic arsenic*
- (b) Pesticides containing cadmium*
- (c) Pesticides containing mercury
- (d) Carbamates
  - (1) Aldicarb (Temik)
  - (2) Carbaryl (Sevin)*
  - (3) Carbofuran (Furadan)****
  - (4) Methomyl (Lannate)
- (e) Fumigants
  - (1) Chloropicrin*
  - (2) Methyl Bromide*
  - (3) Aluminum phosphide
  - (4) Carbon bisulfide
- (f) Mercury treated seeds
- (g) Endrin treated conifer seeds
- (h) Avicides
  - (1) Avitrol
  - (2) Starlicide
  - (3) Strychnine
- (i) Rodenticides
  - (1) Sodium fluoracetate (Compound 1080)
  - (2) Strychnine*
  - (3) Zinc phosphide*
- (j) Organic Phosphorus Compounds
  - (1) Azinphosmethyl (Guthion)
  - (2) Carbophenothion (Trithion)
  - (3) Bidrin
  - (4) Azodrin
  - (5) Monitor
  - (6) Supracide
  - (7) Demeton (Systox)
  - (8) Disulfoton (Di-Syston)*
  - (9) EPN
  - (10) Ethion
  - (11) Methyl Parathion
  - (12) Mevinphos (Phosdrin)
  - (13) Parathion
  - (14) Phorate (Thimet)
  - (15) Phosphamidon
  - (16) Schradan (OMPA)
  - (17) Sulfotepp
  - (18) TEPP
  - (19) Dialifor (Torak)
- (k) Chlorinated Hydrocarbons
  - (1) Aldrin*
  - (2) Benzene hexachloride (BHC)*
  - (3) Chlordane*
  - (4) DDD (TDE)
  - (5) DDT
  - (6) Dieldrin*
  - (7) Endosulfan (Thiodan)
  - (8) Endrin
  - (9) Heptachlor*
  - (10) Lindane*
  - (11) Toxaphene*
- (l) All other pesticides registered for use in the form of a dust, except those products containing only exempt materials.***
- (m) Other Pesticides
  - (1) Paraquat

See next page for explanation of asterisks (*).

TABLE I

RESTRICTED HERBICIDES

Permit required for possession and use except as provided for in Section 2451 (Admin. Code)

(a) 2,4-D
(b) 2,4,5-T
(c) MCPA
(d) 2,4-DP
(e) Silvex
(f) 2,4-DB
(g) Picloram
(h) Propanil
(i)Dicamba (Banvel)

PERMIT REQUIREMENTS

Restricted Materials

* No permit required for home use, structural pest control, industrial and institutional uses, and uses by certain public agencies.

(A) Pesticides containing arsenic other than sodium arsenite as specified in Section 2460 (a) (1)
(B) Pesticides containing cadmium
(C) Pesticides containing mercury
(D) Carbaryl (Sevin)
(E) Chloropicrin
(F) Methyl bromide
(G) Disulfoton (Di-Syston)
(H) Aldrin
(I) Benzene hexachloride (BHC)
(J) Chlordane
(K) Dieldrin
(L) Endosulfan (Thiodan)
(M) Heptachlor
(N) Lindane
(O) Strychnine (Rodenticide uses only)
(P) Toxaphene
(Q) Zinc Phosphide
(R) Pesticides registered for use in the form of a dust included in (1) on reverse side.

** No permit required for ready-to-use syrups or dry baits.

*** No permit required when packaged in containers holding 25 pounds or less or for use in enclosed areas such as greenhouses.

**** No permit required for granular formulations containing not more than 5% carbofuran.

***** No permit required for paraquat for home use only when possessed and used in accordance with registered labeling.

TABLE I

General

(1) Permits to possess restricted materials/herbicides shall not be required of economic poison registrants or pesticide dealers when operating under their licenses, or by governmental agencies or by commercial carriers to transport such materials.

(2) The person in charge of the property to be treated or the pest control operator or both may apply for a permit, but no permit shall be valid for possession or use by any operator or person not named in the permit.

(3) A permit to use restricted materials/herbicides shall have an expiration date no later than the calendar year for which issued and shall be valid for the period specified unless sooner revoked or suspended. A copy of each permit shall be retained by the issuing officer.

(4) The person named in a restricted materials permit is authorized to possess materials for which the permit was valid after such permit expires, provided it is stored in accordance with Section 3136.

REFER TO REGULATIONS FOR SPECIFIC PERMIT REQUIREMENTS.

CALIFORNIA DEPARTMENT OF FOOD AND AGRICULTURE

TABLE II

Field Re-entry Safety Intervals

Safety intervals have been established by regulation between the time certain pesticides are applied to citrus, grapes, peaches, nectarines, and apples, and the time workers may be allowed to enter treated areas to engage in an activity requiring substantial body contact with treated foliage. In addition to the intervals specified by regulation, numerous other safety intervals are included in pesticide labeling and must be complied with. In cases where the safety interval specified in the pesticide labeling differs from the safety interval specified in the regulations, the longer interval must be followed.

Safety Intervals in Days for Citrus, Peaches & Nectarines, Grapes, Apples

	Citrus	Peaches & Nectarines	Grapes	Apples
Azinphosmethyl (Guthion)	30	14	21	14
Carbophenothion (Trithion)	14	14	14	--
Demeton (Systox)	5	7	7	--
Diazinon	5	5	5	--
Dimecron (Phosphamidon)	14	--	--	--
Dimethoate (Cygon)	4	--	4	--
Dioxathion (Delnav)	30	30	30	--
EPN	14	14	14	14
Ethion	30	14	14	--
Malathion	1	1	1	--
Mevinphos (Phosdrin)	4	4	4	--
Naled (Dibrom)	1	1	1	--
Parathion-ethyl	21 (a) 30 (b) 45 (c)	21	21	14
Parathion-methyl	--	21	21	14
Phosalone (Zolone)	--	21	21	--
Imidan	--	5	5	--
Sulphur	1	1	1	--
TEPP	4	4	--	--

Footnotes: (Note these must not be confused with preharvest intervals)

(a) No more than 4 pounds of actual parathion per acre in a single application.

(b) More than 4 pounds of actual parathion per acre, but no more than 10 pounds per acre, in the pat 12 months.

(c) More than 8 pounds of actual parathion per acre per application or more than 10 pounds per acre in the past 12 months.

TABLE III

THE
PROBLEM

Since the introduction of the organophosphate (OP) pesticides into California agriculture in the late Forties, it has become increasingly clear that it is possible to become poisoned from sufficient exposure to residues of these pesticides on foliage and in soils. The population occupationally exposed to such residues is comprised of agricultural fieldworkers engaged in thinning, pruning or harvesting operations. OP-treated citrus crops appear to present a particular hazard but multiple poisonings have also been reported in grapes, peaches, pears, olives and lettuce crops. These incidents led to the imposition of regulations aimed at limiting the exposure of fieldworkers which came into force in the State in 1971 (California Administrative Code, Title 3, Article 2475). However, the mechanisms by which such poisonings occur, the environmental factors involved and the extent of the problem are now being studied.

Incidence of multiple systematic illness from exposure to OP-pesticides for agricultural workers in California, 1949–1974

Date	Location	No. ill	Probable no. exposed	Crop and activity[a]	Pesticide implicated	AIA[b]	Entry time[c]	Previous applications	
								Spray used	Interval[d]
7/8/49	Marysville	20–25	56	Pears	Parathion	2.50	12	—	—
6/27/51	Delano	16	24	Grapes	Parathion	1.87	33	—	—
8/27/52	Riverside	11	30	Oranges	Parathion	2.00	16	—	19
7/6/53	Riverside	7	—	Oranges	Parathion	—	17	—	—
7/ /53	Riverside	—	—	Citrus	Parathion	—	34	—	—
7/ /53	Bryn Mawr	—	—	Citrus	Parathion	—	33	—	—
/ /59	Whole State	275	—	Citrus	Parathion	—	—	—	—
10/5/61	Terra Bella	10	—	Lemons	Parathion	3.00	17	Parathion	97
8/9/63	Hughson	94	—	Peaches	Parathion	2.00	14–38	Parathion	36–110
6/29/66	Terra Bella	9	15	Oranges	Parathion	1.87	15	—	—
7/8/66	Porterville	6	11	Oranges	Parathion	1.33	32	—	—
7/21/66	Lindsay	3	30	Oranges	Parathion	2.00	13	—	—
8/2/66	Navelencia	11	22	Oranges	Parathion– malathion	13.5p	28	—	—
8/11/66	Terra Bella	9	28	Oranges	Parathion– ethion	3.75p	46	—	—
9/2–23/67	Hughson	24	—	Peaches	Azinphosmethyl ethion	1.5a 2.0e	38–47	Dicofol TEPP	38–47 15–30
9/14–16/67	Ballico	3	—	Peaches	Azinphosmethyl	1.5	66	Dicofol	38
5/5/70	Porterville	3	30	Lemons (prune)	Dioxathion naled	6d 1n	1	—	—
5/25/70	Lindsay	2	22	Oranges	Parathion– ethion	7.5p 6.75e	14	Parathion	17
5/27–28/70	Terra Bella	8–11	—	Oranges	Azinphosmethyl ethion	12a 4e	8 11	Azinphosmethyl	10–12
9/14–17/70	McFarland	35	35	Oranges	Parathion	9.00	34–37	Dioxathion	120
10/1/70	Orosi	11	55	Oranges	Parathion malathion	3p	31	Azinphosmethyl	180
8/16–24/71	Orange Cove	8	9	Olives (prune)	Parathion	6.00	31	—	—
5/6/72	Lindcove	3	—	Oranges	Parathion	2.5	21	—	—
9/15/72	Exeter	9	22	Oranges	Parathion	5.00	12	—	—
9/9/72	Huron	4	31	Lettuce (weed)	Parathion	2.50	1	Parathion	4–25
8/30/73	Fowler	27	32	Grapes	?	—	41	—	—

[a] Unless otherwise indicated in "Crop" column, workers are engaged in picking operation. [b] Active ingredient per acre expressed in lb. Days postapplication. [d] Days prior to most recent application.

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DR. D. KURODA: In California, you have many lettuce fields. Suppose, for example, a field is sprayed with a delayed neurotoxin. How is a migrant worker going to know that his onset of symptoms are due to an exposure two weeks ago? Has California had any experience with gathering accident data on delayed neurotoxic pesticides?

DR. J. E. SWIFT: With delayed neurotoxic pesticides, as far as I've been able to find out, and I asked persons who should know about it, -- there are no known cases having occurred in California.

If you're asking about leptophos, yes it was used, but its usage has been stopped for the present time. EPN is a material that is no longer used. You can hardly even buy it in the state anymore. It did not prove to be effective as a pest control agent. With most of these pesticides I'm talking about -- at least the organophosphates and the carbamates, if they're used on a field, the field has to be posted in both English and Spanish in letters that can be read at least 25 yards away. It's posted on all four sides of the field and the entrance place. Until the re-entry period that has been settled upon has passed. After that they have to be taken down. This applies to any sort of a crop, if certain compounds are used. EPN was put on this category where it had to be used in posted fields. This was done as long ago as the early 1950's. EPN is just not used anymore.

DR. W. J. HAYES: I think this has been a very valuable contribution to hear about these occupationally connected cases from California. These statistics have been available for many years. There's nothing quite comparable to them in any of the other states. So, it's very valuable to hear these reports of, in most instances, very mild effects, with reporting stimulated by a compensation system.

I thought it might be of interest to you, since I've had to look into it, to get a little perspective on how this compares with the other parts of the country and the world, and even with California itself. In other statistics issued by the state, but published separately for the last several years, to give you some idea of the kind of thing that they will record, the number of cases of poisoning by poison ivy is considerably in excess of those by pesticides.

Now, in other states and countries where they count only those accidents that are of a more serious nature, and, at least in the state of New York, are compensable, what one finds is that the number of agriculturally related injuries is very much higher for equipment, and even hand tools, than it is for pesticides. The number of pesticides used are really quite small. If you take that kind of a base, then you get essentially identical statistics from places as far removed as New York State, the United Kingdom and Hungary. I have been searching for some years to get comparable figures from California, but without success.

PESTICIDE REGULATORY RESPONSIBILITY

Orville E. Paynter, Ph.D.

Perhaps the only contribution a paper on pesticide regulatory responsibilities might make to these proceedings is to give some insight into the growing requirements of regulatory agencies for scientific input and indicate how this has come about. The extent of the authority exercised by such agencies is governed by the way elected officials interpret society's needs and take action through enactment of appropriate laws. In the past pesticide regulation had proceeded at a leisurely pace, laws were not very complex and the scientific input required to make them effective was minimal. Today this is no longer true.

Federal Regulation of pesticides in the U. S. began with the Insecticide Act of 1910 which prohibited the Interstate sale of any insecticide or fungicide which was adulterated or misbranded. This Act was concerned with the effectiveness of products and deceptive labeling and did not require registration of pesticides. It can be seen that the concepts of pesticide regulation were not very complex and the scientific input, if any, was confined to rather simple chemical, entomological and perhaps microbiological considerations.

In 1947, 37 years later, The Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) established registration requirements for all pesticides entering interstate trade, but authority to deny registration applications was not provided until 1964. As with the 1910 Act, FIFRA's primary thrust was the protection of consumers from ineffective products and deceptive labeling. However, FIFRA did require that pesticide labels contain precautionary warnings and statements which would protect the consumer from certain adverse effects. For the most part, these warnings and statements were concerned with the acute toxic effects to humans and domestic animals. The law had become more complex as society's perception of the problems had developed and changed. In addition to the scientific input of previously mentioned deciplines, the inputs from pharmacology, toxicology and various health sciences were now required to make the law effective.

In 1972, FIFRA was drastically amended by the Federal Environmental Pesticide Control Act (FEPCA). Additional amendments also occurred late in 1975. Congress found the greatest need for the 1972 revision of FIFRA to be in the areas of strengthening regulatory controls on the uses and users of pesticides, speeding up procedures for barring pesticides found to be undesirable, stremlining procedures for making valueable new measures, procedures, and materials broadly available; strengthening enforcement procedures to protect against misuse of pesticides; and creating an administrative and legal framework under which continued research could provide more knowledge about better ways to use existing pesticides as well as developing alternative materials and methods of pest control. These amendments, 65 years after the first law to control pesticides, produced a complete metamorphasis of the Federal pesticide regulatory scheme and completely redefined its thrust. FIFRA was changed from a labeling law into a comprehensive regulatory statute controlling the manufacture, distribution and use of pesticides. While FIFRA as amended is still concerned with the older concepts of 1910 and 1947, that is the effectiveness of pesticides, deceptive labeling and registration of pesticides; its thrust is primarily oriented toward the protection of man and the environment from unreasonable adverse effects caused by pesticide use. This orientation produced a reordering of priorities in the sense that human health effects and environmental effects are of primary concern in pesticide registration and the regulatory actions taken by EPA.

Two other laws also provide safeguards for the protection of man from potential long term or chronic adverse effects of pesticides and add their authority to controlling misuse by requiring the establishment of pesticide tolerances and providing enforcement procedures. Federal regulatory control of pesticide residues in food and animal feeds is exercised through the Federal Food, Drug and Cosmetic Act (1938) primarily administered by the Food and Drug Administration (FDA). EPA has the responsibility for establishing the pesticide tolerances and FDA's responsibility is that of enforcement. Like FIFRA, this Act has undergone a series of amendments over many years and has progressed from concerns with adulteration and misbranding to regulation and control of useful chemicals in the food supply. The Pesticide Amendment of 1954, which inserted Sec. 408 into the Act, provided a simplified mechanism for the establishment of tolerances for pesticide residues in or on raw agricultural commodities. In an important departure from previous food legislation, it provided only that the additive pesticide be certified as useful (not required) in production of the food. A

Federal Register notice of March 11, 1955, contained a large number of tolerances established under authority of Sec. 408 arising from the findings of fact from a 1950 Spray Residue Hearings. All subsequent tolerances were pursued through individual pesticide petitions as prescribed in the Procedural Regulations for Sec. 408. The 1958 Food Additives Amendment inserted Sec. 409 into the FFDC Act. This Amendment provides extremely broad regulatory coverage to additives in foods but its important impact on pesticides is that it provided the mechanism to regulate pesticide residues in processed foods to complement the authority under Sec. 408 on raw agricultural commodities.

The Meat and Poultry Inspection Act is administered by the U. S. Department of Agriculture. The act does not itself provide for the establishment of pesticide residue tolerances in meat or poultry. It does, however, provide enforcement procedures for compliance with tolerances for pesticides in meat and meat products established by EPA under the applicable provisions of the FFDC Act.

This very brief history of pesticide regulatory authority should provide some insight into the various kinds of regulatory responsibilities shared by many individuals within EPA and other agencies interested in pesticide problems. The scientific inputs required to effectively administer the amended FIFRA and establish tolerances are enormous and are seriously straining the ability of the Registration Division's technical resources to keep pace with developments impacting mandated responsibilities.

Perhaps the individuals most affected by the reorientation of priorities toward human and environmental effects, are those individuals we might call regulatory toxicologists and environmental hazard evaluators. These designators should be interpreted in the broadest sense, since many diverse scientific disciplines are involved. These individuals bear the responsibility for examining and evaluating pesticide data submitted to them from various sources; rendering an opinion as to the adequacy and scientific integrity of the submitted data; making a judgment as to the relevance of the data to the perceived risks to human health and the environment; and ultimately recommending some type of regulatory action. It is obvious that the quality of the judgments made and the quality of the regulatory actions recommended are dependent upon the adequacy of the submitted data, the adequacy of the available data base to which the submitted data are compared, and the training, competence, and experience of the individuals involved. Therefore, the continued development of adequate data bases by basic research in various health and environmental disciplines, the sharing of opinions as the present state of the art (as we have been doing at this conference) and the strengthening of benefit/risk analysis are essential if society is to benefit, in the long run, from pesticide regulatory decisions.

A second important insight which the brief history of regulatory authority should produce, is that while the regulatory scientist endeavors to render judgments and opinions consistent with his training, experience and the available data base, he is not entirely free of the legal influences which are an essential part of a regulatory agency's mandate and environment. For this reason, he is frequently involved in actions of regulatory importance even though the basic sciences have not yet provided a firm base for sound regulatory policy or action. This is especially true in the broad and complex areas of environmental effects, carcinogenicity, and mutagenicity. These examples are by no means exhaustive, since the subject of this conference falls into the same category and more examples could be put forward. When society demand regulatory action under these circumstances, the vacuum is filled somehow, the results are usually controversial to say the least, and the action taken might be inadequate in the long run.

This conference was planned nearly a year ago to prevent, if possible, this type of situation from occurring in the area of delayed neurotoxicity. It is of course too early to judge the results in this respect, but I believe that it has been successful in several ways. While gross observations for neurotoxicity are an integral part of almost every toxicological study, this conference reemphasized the fact that the various species generally used in these tests are not especially sensitive indicators of delayed neurotoxicity. It has indicated that while knowledge of the possible mechanisms of action for some organophosphates has increased, the techniques, procedures and test systems relied upon by regulatory agencies in many respects have remained static. More important than these benefits, this conference has gone a long way in satisfying the regulatory needs for procedures which can measure dose effect relationships. Still to be addressed is the questions of potential cumulative effects of long term, low level exposure such as might be received through the food supply or from long term occupational exposure. It is possible, by application of the results of this conference, that regulatory scientists can arrive at an intelligent solution of this problem. As many of you know, the EPA is currently revising the Guidelines for Registering Pesticides in the U. S. The results of this conference could improve the Methods for Study of Neurotoxicity section in the Appendix of the Guidelines in a significant way. We in the sponsoring organizations thank each of you for these contributions.

DR. M. K. JOHNSON: I have been questioned quite a bit about what one does about translating from the fundamental investigations to the regulatory aspects. I have one suggestion which I think might be worthy of being discussed, a propos of guidelines which were discussed. I think it requires that we accept three presuppositions. First, we must accept that the acute effects of organophosphate insecticides are basically a reflection of their anti-cholinesterase activity, and also that the delayed neurotoxic effects, as and when they appear, are a function of that anti-neurotoxic esterase activity. Second, we must accept that some sort of test on hens is going to be worthwhile, and last, we accept that for most OP pesticides, the active antiesterase molecule is in fact the oxon. I realize this, perhaps for some materials, may be a problem, but for a majority I think we would accept that the active anti-esterase molecule is the oxon. Could I suggest then that, as a minimum, it would be worthwhile baseline information to measure, in vitro, the activity of the appropriate oxon against acetyl cholinesterase and neurotoxic esterase, presumably in hen brain or some appropriate tissue just to have those numbers in front of one when thinking about neurotoxic potential.

I make no suggestion as to whether one number should be larger than the other or what one does about the fact that from cholinesterase poisoning there is a chance of recovery and treatment with atropine and PAM, and with neurotoxicity there doesn't seem to be much chance. But it seems to me to have those numbers available for intelligent thinking might well be useful.

DR. O. E. PAYNTER: I think that what you have just said, is a significant contribution to this meeting. I think that's the way that regulatory agencies would want to go although I would have one caution. Numbers, in some places, become quite holy and sacred and set in concrete. I noted yesterday, you made a plea that hopefully, some regulatory agency wouldn't set some level and say, anything above this is banned, nasty, or something else. Hopefully that won't happen. But, I suggest that it may. I would not discourage anyone from using these two systems, the hen test and the particular test system that you are recommending. I think that's the way to go.

DR. J. E. CASIDA: I think the suggestion is quite a useful one, but the number of compounds that would have to be tested will be quite large when you consider, in some of the commercial compounds, all of the metabolites that contain anti-esterase activity and all of the photo products that do the same. So, that it might not be quite as simple, as they discovered in mutagenesis tests, where they had to make ramifications to accomodate such features.

DR. W. UPHOLT: Ladies and Gentlemen, thank you for your comments and discussion. My thanks also to the speakers for their excellent presentations. There are a number of conclusions that can be drawn from these two days. But what I prefer to think is that in order to avoid serious pitfalls, we must be constantly alert to the hazards, and also must have a better understanding of cause and effect. You have helped us by your participation in this conference, over these categories, and we do appreciate it.

I want to especially thank those distinguished scientists who have come from abroad. They have added immeasurably to this conference.

If there are no more comments, we stand adjourned, and thank you very much for participating.

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